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A SIMPLE RECORDING IMPULSE COUNTER

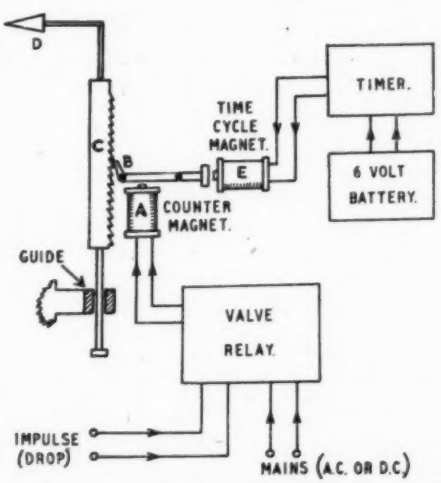
BY

R. H. THORP

From the Wellcome Physiological Research Laboratories, Beckenham, Kent

(Received March 27, 1948)

During an investigation of the action of drugs upon the isolated mammalian heart by means of the Langendorff preparation, it was observed that certain substances produced a marked acceleration in the rate of the heart beat. In order that such observations could be recorded, an impulse counter was designed capable of making a permanent record on a kymograph.



SCHEMATIC DIAGRAM OF RECORDING IMPULSE COUNTER.

FIG. 1.

The principle of the impulse recorder is shown diagrammatically in Fig. 1. Each impulse operates the electromagnet *A* and draws down the pawl *B*. When the circuit is broken at the end of the impulse, the return of the armature pawl lifts the rack bar *C* by one tooth. There is a second pawl, concealed by *B* in the diagram, which prevents the rack bar from falling while the pawl *B* is in motion.

The rack bar is cut to give 50 teeth each of one millimetre pitch and a writing point *D* is attached to the top of the bar. Impulses are therefore recorded by a line which rises 1 mm. per impulse on the kymograph drum and may be a stepped diagonal line on a fast moving drum, or a virtually vertical line on a slow moving drum. The impulse count is recorded over timed intervals which can be derived from any time marker clock used in physiological laboratories. At the end of the counting cycle an impulse from the time clock energizes the electromagnet *E* which pulls back the complete armature and pawl assembly so that the rack bar is reset to the base line.

The electromagnet used for counting the impulses is wound to a resistance of 1,500 ohms and needs an operating current of 15 milliamps. This has been so constructed for convenience in operating the device from a valve relay of the pattern described by Winton (1936, 1939).

The time cycle release magnet coil is wound to a resistance of 4 ohms and operates on a 6 volt supply, but could be arranged to operate at mains voltage if necessary.

The photograph Plate I shows the construction of the device and the same lettering has been used as in the diagram. The two projecting screws *F* and *G* enable the counter to be set or released manually.

In recording the rate of the isolated heart the impulse was derived from two contacts on the lever recording the individual beats; these contacts were connected to the valve relay unit.

The counter can be used for recording a drop outflow with very satisfactory results, by using a silver drop tube of the pattern mentioned by Winton (1936), and connecting this to the valve relay.

Several workers have described instruments of this type, but the earliest is probably that of Negrin (1919) followed by the instruments of Fleisch and later that described by Gaddum (1938).

It is common experience in the science of measurement that several instruments are neces-

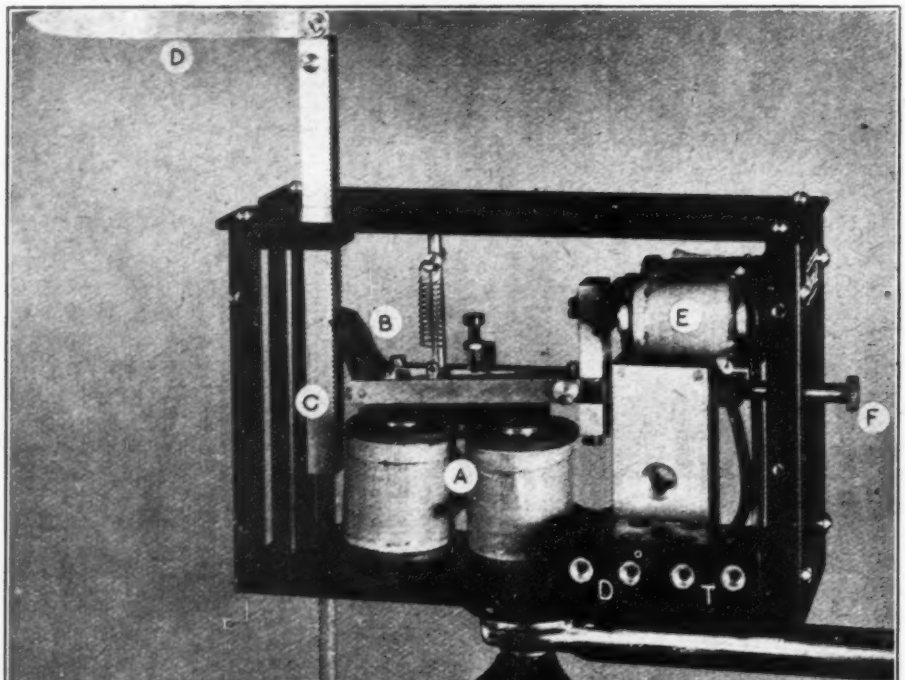


PLATE I.—Recording impulse counter with cover removed to show mechanism.

sary to cover a wide range of variation in a given function. Gaddum's device records on the kymograph the time elapsing between two successive impulses, and is excellent when these impulses are several seconds apart, but unsuitable for impulse rates faster than 100 per minute. The instrument at present described counts the impulses and by alterations in the slope of the recorded trace gives an indication of a change of interval between impulses within any one timing cycle, which can be of any length greater than 5 seconds. The fastest rate the present model can record is between 550 and 600 impulses per minute.

The applications of such a device are many, and its fundamental advantage is its inherent linearity and the ease with which the individual impulses can be counted on the record.

I should like to thank Mr. Ellis, of Messrs. C. F. Palmer (London), Ltd., who constructed the instrument illustrated from a simple prototype.

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SYSTEMIC EFFECTS OF ADENOSINE TRIPHOSPHATE

BY

N. EMMELIN* (Lund) AND W. FELDBERG†

From the Physiological Laboratory, Cambridge

(Received April 6, 1948)

The experiments described in the present paper were the outcome of an incidental observation made in decerebrate cats when adenosine triphosphate (ATP) was injected into the artery supplying a leg muscle, the tibialis anticus. These injections were intended to produce the direct muscle contracting action of ATP first described by Buchthal and Kahlson (1944). We started with small doses of ATP which gave no contractile response; the dose was then increased with the result that sometimes, but by no means always, immediate weak contractions could be recorded from the tibialis anticus, but the injections produced additional and more striking effects. After a latency of some 30 seconds, the time necessary for the ATP to reach the general circulation after having passed through the tibialis anticus muscle, respiratory changes occurred, followed by strong contractions of the skeletal muscles of the limbs and the whole body of the animal. ATP is known to produce profound changes in the cardio-vascular system (Drury and Szent-Györgyi, 1929; Gaddum and Holtz, 1933; Gillespie, 1934; McDowall, 1944; Bielschowsky, Green and Stoner, 1946) and the possibility was therefore envisaged that these general effects might be the outcome of circulatory events.

The present paper deals with circulatory, respiratory, and other systemic effects of ATP when injected into different parts of the circulatory system.

METHODS

Most experiments were performed on cats, decerebrate or in chloralose anaesthesia; a few experiments were performed on dogs and rabbits also anaesthetized with chloralose. The systemic arterial blood pressure was recorded from the left external carotid or the femoral artery with a mercury manometer. In the same way the pressure in the pulmonary artery was recorded in some cats as well, the artery of the lower lobe on the left lung being cannulated in this case. The heart rate was read from the tracing of the systemic blood pressure; for this purpose the drum was run at a fast speed.

For intravenous injections of ATP cannulae were tied into a jugular or femoral vein. When the ATP was intended to reach the systemic, without first passing the pulmonary circulation, it was injected either into the left auricle or through a glass cannula tied into the left auricle or through a long fine syringe needle, with a blunt tip, which was passed through an opening in the right external carotid or subclavian artery down to the base of the ascending aorta. Heparin was often given in these experiments. For injecting ATP into the brain circulation it was injected into the vertebral or external carotid artery. For the injections into the vertebral artery again a fine blunt needle attached to a syringe, filled with the amount of ATP to be injected, was passed through an arterial side branch into the vertebral artery. In order to ensure that all the injected ATP passed down the vertebral artery the vessel was sometimes tied over the needle during the injection, but immediately afterwards the ligature was loosened and the needle withdrawn so that the blood supply to the vertebral artery was restored. When ATP was injected into the external carotid, the carotid sinus region was usually denervated and the injections were made through a cannula tied into the lingual artery pointing toward the carotid, which during the injections was kept clamped at its aortic end with an arterial clip.

When we began injecting ATP into the left coronary artery we used cats and introduced a fine blunt syringe needle, slightly bent at its end, through an opening in the subclavian artery into the ascending aorta and then guiding it by touch into the mouth of the left coronary artery. It was either kept here without further fixation during the injection or tied with a thread previously placed loosely round the origin of the coronary artery. In these experiments the left coronary artery is supplied with blood by collaterals only. Later on we used, in cats and dogs, the methods described by Dawes for cats (1947). With this method the circulation through the left coronary artery is not interrupted. A specially shaped glass cannula is required for these experiments; it was kindly supplied to us by Dr. G. S.

* With a grant from the Swedish Medical Research Council.
† With a grant from the Medical Research Council.

Dawes (Oxford). In all experiments with injections into the left coronary artery heparin was used.

Respiration was recorded by a modification of Gaddum's (1941) method. The trachea was attached to a glass tube with inflow and outflow valves, which gave as little resistance to respiration as possible. The expired air was blown into a 10 litre bottle, from which a fine glass tube led to the outside air and a wide tube to a sensitive tambour for recording the respiratory changes. Unlike Gaddum's method this arrangement does not provide a quantitative record of the respiratory changes, but it serves the purpose of recording easily changes in rate and depth of respiration. Inspiration is shown in the tracings as a downward, expiration as an upward stroke.

In some experiments it was desired to exclude vagal impulses for short periods. For this purpose the vagi were cooled at the neck by placing them each on a separate 1.5 cm. wide thin copper plate, which was slightly bent at its end in order to prevent the nerves from slipping off the plate. The plates were 16 cm. long and kept cold during the period of cooling the nerves by placing the free ends into beakers full of crushed ice. In addition a little metal box was soldered on each plate near the end provided to take the nerve and also filled with crushed ice.

Three different preparations of the barium salt of ATP were used. One sample was prepared by ourselves by a modified version of Lohmann's method (1931), one sample was kindly supplied to us by Professor F. Buchthal (Copenhagen), and a third sample was obtained from Boots' Drug House. Before use the Ba salt was converted into the sodium salt. When the three preparations were compared for their ability to activate the synthesis of acetylcholine in extracts of acetone dried brain tissue the sample obtained from Boots was found to be only 80 per cent as active as the other two samples, which had practically identical activity. No obvious difference was observed with the three preparations on injections into the circulations of cats or dogs; a 20 per cent reduction in activity, however, would not have been noticed in these experiments. In the text the dose of ATP injected has been expressed as ATP pyro—P (ATP—P). In order to obtain the value for the total phosphorus injected the value has to be multiplied by 1.5. In a few experiments control injections were made with creatine phosphate which was kindly prepared for us by Dr. P. Eggleton and Dr. Nimmo Smith as the synthetic Ba salt and converted before use into the Na salt. The values for creatine phosphate are expressed in the text as mg. total labile phosphorus (CrP—P), about 1 mg. of which is present in 12 mg. of the Ba salt.

Results

Circulatory effects in cats

The intravenous injection of 0.2 to 0.4 mg. of ATP—P causes a profound and steep fall in arterial blood pressure (Fig. 1). During the fall the heart pulsations on the blood pressure tracing become feeble or the float on the mercury manometer may even write a smooth line on the smoked drum. When the oscillations are not obliterated, pronounced slowing of the heart can be seen on the blood pressure tracings. In Fig. 1a the steep fall in arterial blood pressure is associated with an initial period in which the heart oscillations are no longer visible on the tracing; later pronounced slowing of the heart will be seen. In the experiment of Fig. 1b the blood pressure tracing gives during the whole period of the depression a smooth line.

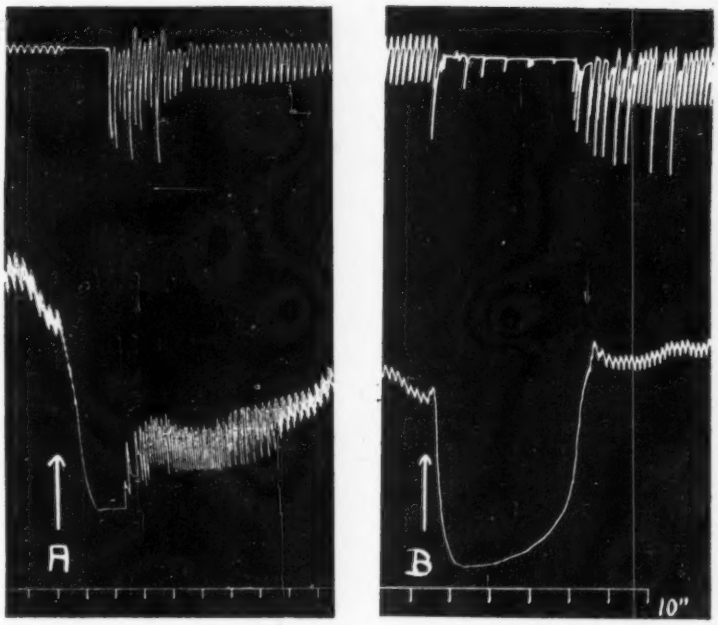
When the blood pressure has recovered a renewed injection of ATP produces approximately the same strong fall in arterial blood pressure as the first injection, but on further repetition of the injections the sensitivity to ATP gradually decreases, so that it has to be given in increasing doses in order to elicit responses as strong as the initial ones.

Other organic as well as inorganic phosphate compounds injected in doses containing the same or even a greater amount of total phosphate than that injected with the ATP, produced either no fall in arterial blood pressure (for instance 0.6 mg. CrP—P, 7 mg. sodium triphosphate or 15 mg. sodium pyrophosphate) or a fall uncomplicated by slowing of the heart or by reduction in the heart oscillations (for instance 10 mg. muscle or yeast adenylic acid).

The following factors are responsible for the depressor action of ATP in cats: (*a*) obstruction in the pulmonary circulation, causing a reduction in cardiac output, (*b*) reflex slowing of the heart causing further reduction in cardiac output, and (*c*) vasodilatation.

Obstruction in the pulmonary circulation.—In order to observe the effect of ATP on the pulmonary circulation it is best either to cut the vagi or to give atropine in order to eliminate the slowing of the heart. Under these circumstances ATP still produces its pronounced depressor effect, showing that the slowing of the heart observed in the absence of atropine and with the vagi intact is not the sole or main factor responsible for the fall in systemic blood pressure produced by ATP.

The injection of 0.2 to 0.4 mg. ATP—P into the jugular vein or into the right auricle of a cat with the thorax open, artificial ventilation and the vagi cut or atropine given, produces tremendous swelling of the pulmonary artery and of the whole right heart, ventricle and auricle; the left remains small. This can be observed with the naked eye in a cat with open thorax and artificial ventilation. These volume changes do not take place when the



(a) (b)

FIG. 1.—Respiration (upper) and arterial blood pressure (lower) tracings of decerebrate cat (*a*) and cat under chloralose (*b*). At A, 0.2 mg., at B, 0.4 mg. ATP–P intravenously. Time in 10 seconds.

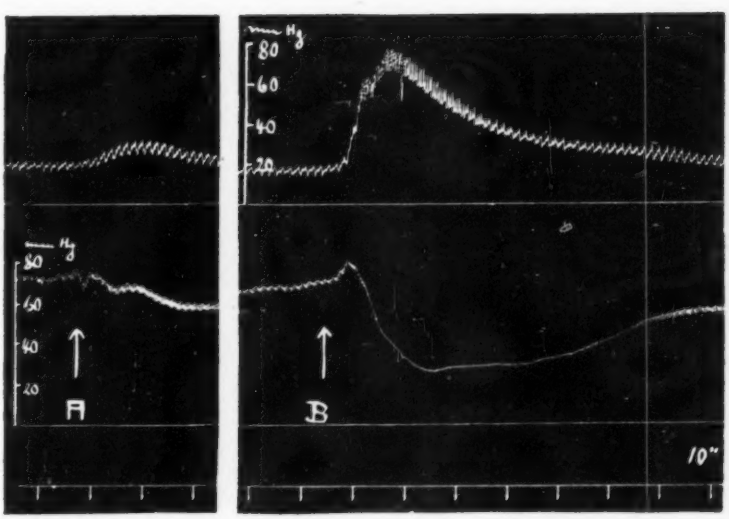


FIG. 2.—Pressure in pulmonary artery (upper tracing) and carotid (lower tracing) of 2.2 kg. cat under chloralose. Thorax opened ; artificial ventilation ; both vagi cut. At the arrows 0.2 mg. ATP–P into the left auricle (at A) and into the jugular vein (at B). Time in 10 seconds.

same dose of ATP is injected into the left auricle and reaches the pulmonary artery greatly diluted after its passage through the systemic circulation. Incisions into the carotid arteries, when the intravenous injection of ATP had produced its characteristic fall in arterial blood pressure with elimination of the heart oscillations and the typical changes in heart volume, caused very little bleeding, whereas a subsequent incision into the right auricle or the superior vena cava produced a rushing out of blood into the thorax. All these observations are accounted for by a strong obstruction in the pulmonary circulation whereby the blood is prevented from reaching the left heart; consequently the cardiac output drops.

In Fig. 2 the pulmonary obstruction produced by ATP is shown by recording its effect on the pressure in the pulmonary artery simultaneously with the carotid blood pressure. Usually the opening of the thorax and tying a cannula into a pulmonary artery and into the left auricle lowers the carotid blood pressure to about 100 mm. Hg or to an even lower level. In this condition ATP apparently produces little further vasodilatation, as seen from the fact that an injection of 0.2 mg. ATP—P into the left auricle causes scarcely any further fall in carotid blood pressure (Fig. 2,A). The pressure in the pulmonary artery also is little affected by this injection of ATP. The result, however, is different when the same dose of ATP is injected intravenously (at B); the pressure in the pulmonary artery rises immediately and steeply from its initial level of about 20 mm. Hg to about 80 mm. Hg. The pressure in the carotid artery starts to fall a second or two after the beginning of the rise in the pulmonary artery and then follows it in reverse direction. During the fall in systemic blood pressure the heart oscillations on the carotid pressure tracing become feeble.

With repeated intravenous injections of the same dose of ATP the effect on the pulmonary blood pressure becomes gradually smaller and so does the fall in systemic blood pressure. But an increase in the dose of ATP produces again the strong pressure changes in the pulmonary and carotid artery.

Vasodilatation in the systemic circulation.—We have seen that in cats in which a cannula has been tied into the pulmonary artery the arterial blood pressure often falls to a level of about 100 mm. Hg or lower owing most likely to a decrease of tone in the systemic vessels. In such cats no evidence could be obtained for a vasodilator action of ATP. The result, however, is different in those cats in which the level of the arterial blood pressure has remained high even after opening the thorax and tying a cannula into the left auricle; in these conditions ATP in doses too small to produce a fall in arterial blood pressure, when injected intravenously, has such an action when injected into the left auricle. In the experiment of Fig. 3 a dose of 0.05 mg. ATP – P injected intravenously

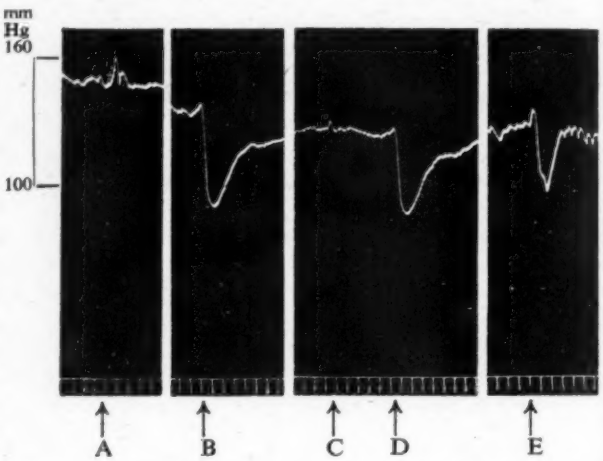


FIG. 3.—Carotid blood pressure of 3.2 kg. cat under chloralose. Both vagi cut; thorax opened, artificial ventilation. At A and E intravenous injection of 0.05 mg. and 0.2 mg. ATP–P respectively. At B and D 0.025 mg. ATP–P and at C 1 c.c. saline injected into the left auricle. Time in 10 seconds.

at A did not lower the arterial blood pressure, but half the amount injected into the left auricle, at B and D, caused an evanescent but pronounced fall, which in fact was stronger than that produced by eight times the dose of ATP injected intravenously at E. Vasodilatation in the systemic circulation therefore also contributes to the usual depressor action of intravenous injections of ATP.

No experiments have been carried out to analyse the vasodilatation. It is unlikely that reflex vasodilatation occurs with ATP when the vagi nerves have been cut. This may happen, however, in cats with intact vagi which could carry afferent impulses to the vasomotor centre. The effect would then resemble that of veratrin (Dawes, 1947). Our experiments also do not deal with the possibility of a centrally induced vasodilatation. Such an effect cannot be excluded by our finding that ATP, at least in cats under chloralose anaesthesia, has no central cardiac action.

Slowing of the heart.—The pronounced slowing of the heart when ATP is injected intravenously is dependent on the integrity of the vagi. This had been observed by McDowall and by Bielschowsky, Green, and Stoner (1946). They had concluded that ATP stimulates the vagus centre in the medulla.

Our results, however, show that the slowing is mainly accounted for by a reflex, the afferent and efferent impulses of which are carried in the vagus nerve. In fact in cats under chloralose anaesthesia the bradycardia obtained on injections of 0.2–0.4 mg. ATP – P is solely accounted for in this way. In decerebrate cats, on the other hand, stimulation of the cardio-inhibitor centre by ATP probably contributes to the bradycardia. In these cats injections of 0.2 to 0.4 mg. ATP – P into a vertebral artery slow the heart, but in cats under chloralose neither an injection into a carotid artery, in central direction, nor into a vertebral artery was effective, whereas the same amount of ATP injected into the left auricle produced pronounced bradycardia even when the carotid and vertebral arteries were occluded during the injection. It might be mentioned in this connexion that clamping of the vertebral arteries in decerebrate cats, but not in cats under chloralose, also led to bradycardia. The reflex nature of ATP-bradycardia in cats under chloralose anaesthesia is illustrated by the experiment (Fig. 4) in which 0.1 mg. ATP – P was injected either into the right vertebral artery (at B) or into the left auricle (at A). The injection into the auricle reduced the heart rate from 34 per 10 seconds to 9 per 10 seconds, whereas the vertebral injection did not change it. Between B and C the carotid and vertebral arteries were clamped ; this caused a rise in blood pressure but no slowing of the heart. When the blood pressure had reached a relatively steady level 0.1 mg. ATP – P was again injected into the left auricle, at C. The bradycardia was not prevented by having excluded the brain circulation, but was abolished after cutting the vagi. In another experiment on a cat in chloralose anaesthesia 0.1 mg. ATP – P was injected into the right carotid, whilst it was kept clamped for a few seconds at its aortic end, or into the left auricle, the carotid and vertebral arteries being kept occluded for about 10 seconds before and after the injection. Again only the injection into the left auricle caused slowing of the heart (from 3.4 to 1.5 beats per second) despite the fact that the pathway to the brain was occluded, but the bradycardia was abolished after cutting the vagi. This experiment shows in addition that the reflex does not originate from chemoreceptors in the carotid body, a fact which has been verified in cats with both carotid sinus nerves ligated and cut. These experiments do not exclude an action of ATP on the chemoreceptors in the aortic body, but it would be strange if they were, and those in the carotid body were not, sensitive to ATP. The region of the aortic arch, however, has not been excluded as a contributory area from which the reflex could be elicited although, to some extent at least, it originates in the heart itself and thus resembles the bradycardia produced by veratrin alkaloids (Dawes, 1947) ; this was evident when ATP was injected into the coronary artery.

In one cat we succeeded in tying a needle cannula, introduced through the subclavian artery, into the main branch of the left coronary artery without causing ventricular fibrillation. Fig. 5 is taken from this experiment ; the injection of 0.5 c.c. of saline into the coronary artery had no effect (at A), but 0.1 mg. ATP – P slowed the heart rate from 28 per 10 seconds to 13 per 10 seconds. The effect of ATP could be obtained with each renewed injection, but only as long as the vagi were left intact. A post mortem injection of Chinese ink through the cannula into the coronary showed that it supplied practically the whole left heart. There must, however, have been effective anastomoses with other coronary branches which kept the muscle supplied with blood. Some indication of these was obtained during the experiment by the fact that there was a strong back flow of blood whenever the syringe attached to the needle was removed after an injection. In later experiments Dawes's method was used in which the coronaries are continuously supplied with blood. In these experiments also the injections of ATP into the coronary circulation caused slowing of the heart, but not after cutting the vagi. Doses larger than 0.2 mg. ATP – P were not tried. The slowing produced with the vagi intact was often less pronounced than that following the injection of a similar dose of ATP low down into the ascending aorta. This difference may be due to the fact that only part of the heart area from which the reflex is elicited will be reached when ATP is injected through the cannula tied into one coronary or that some receptors for this reflex are situated in the aortic arch.

Slowing of the heart by ATP in rabbits and dogs. —Our results obtained in *rabbits* are in agreement with those of Bielschowsky *et al.* (1946) who found that in these animals ATP has a direct cardiac depressant action. The injection of ATP into the left auricle caused pronounced bradycardia which was not prevented by cutting the vagi or by atropine. The effect was evident with 0.05 mg. ATP – P and became stronger with larger doses, but on repeated injections the slowing became gradually less pronounced.

In *dogs* the injections of ATP into the left auricle or down to the base of the ascending aorta also caused pronounced slowing of the heart, which, like that seen in cats, was prevented by

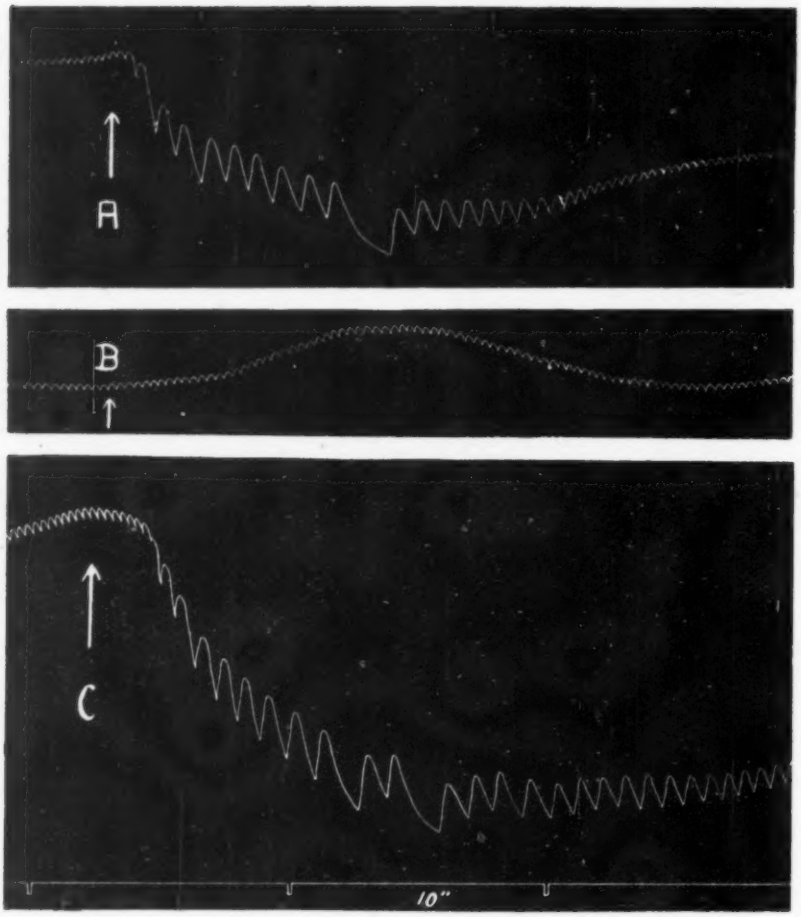


FIG. 4.—Carotid blood pressure of 3.2 kg. cat under chloralose; thorax open. Artificial respiration. At A and C 0.1 mg. ATP-P injected into left auricle, at C with carotid and vertebral arteries occluded. At B 0.1 mg. ATP-P injected into left vertebral artery. Time in 10 seconds.

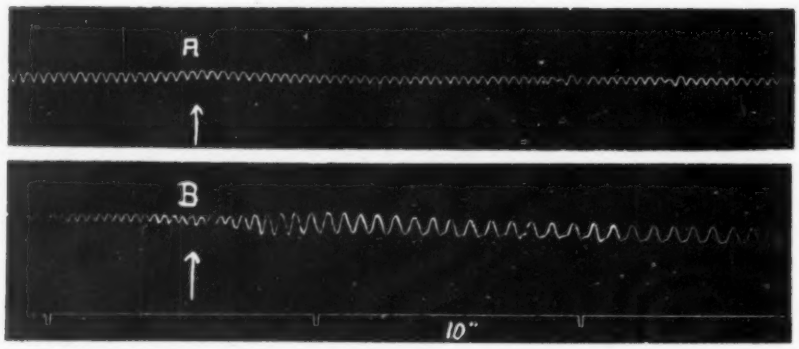


FIG. 5.—Blood pressure from femoral artery of 2 kg. cat under chloralose. Thorax open. Artificial ventilation. Heparin. Cannula tied into left coronary artery through subclavian artery. At A injection of 0.5 c.c. saline, at B of 0.1 mg. ATP–P into coronary artery. Art. blood pressure about 60 mm. Hg. Time in 10 seconds.

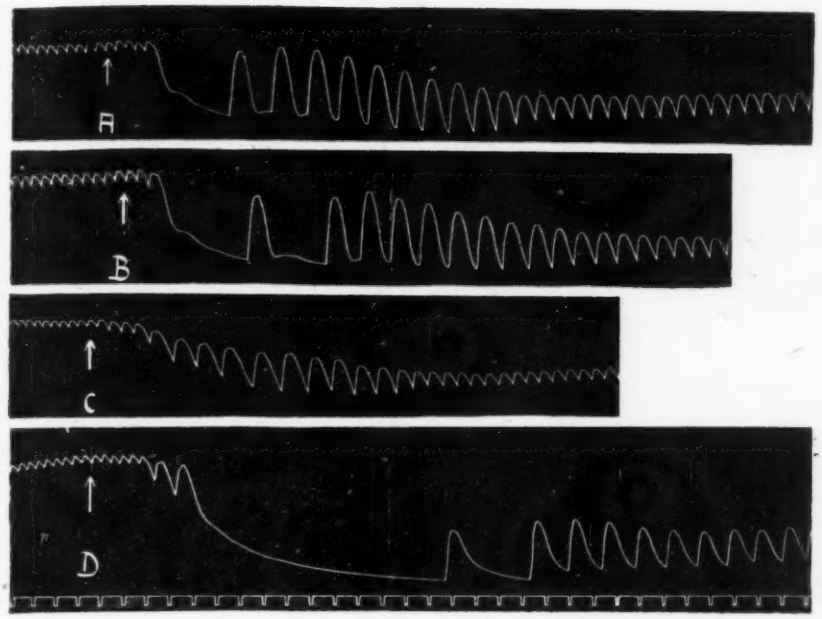


FIG. 6.—Carotid blood pressure of 11 kg. dog under chloralose. Thorax open; artificial ventilation. At A and B injections of 0.2 mg. ATP–P into ascending aorta at its base and at C and D into right vertebral artery. At B carotid and vertebral arteries occluded. Time in seconds.

cutting the vagi or by atropine. The effect was partly reflex, partly central in origin. In the experiment of Fig. 6, for instance, 0.2 mg. ATP – P injected low down into the ascending aorta produced pronounced bradycardia whether the carotid and vertebral arteries were left open (at A) or clamped (at B) before and during the injection. The effect therefore cannot be wholly due to stimulation of the cardio-inhibitor centre by ATP. The participation of a central factor, however, was shown by the bradycardia obtained when the same dose of ATP was injected into a vertebral artery; the slowing of the heart was weaker at C but stronger at D than that produced by injections into the left auricle at A and B. These doses of ATP had certainly no direct cardiac depressant action since they were ineffective after cutting the vagi. In another experiment central cardiac inhibition was obtained by injecting 0.2 mg. ATP – P into the carotid through a cannula in the lingual artery whilst the carotid was clamped at its aortic end. Ligating and cutting the sinus nerve did not abolish or diminish the effect, but if, subsequently, the vessels from the carotid leading to the brain were tied ATP became ineffective. The reverse procedure was adopted in the experiment shown in Fig. 7; the bradycardia became nearly abolished after the blood vessels to the brain had been tied, although the sinus nerve was kept intact. This was done between A and B. The slight slowing seen at B is probably not due to ATP, since saline injections into the carotid artery with its lower end occluded produced the same effect. The injections probably increased the pressure in the carotid artery for a short period and consequently stimulated the pressor receptors.

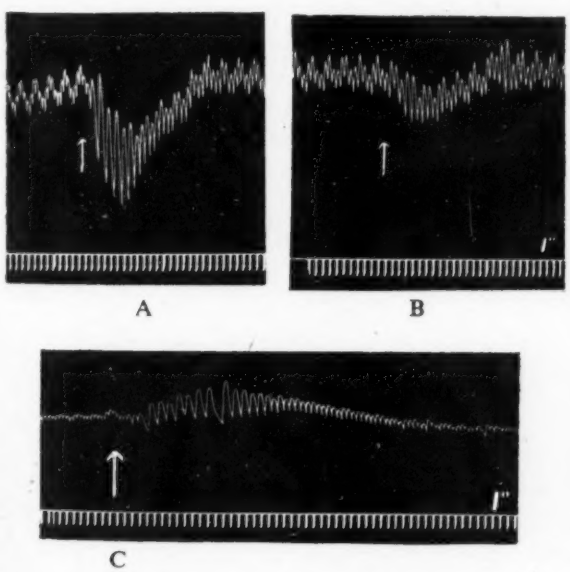


FIG. 7.—Arterial blood pressure of 8 kg. dog under chloralose. Injections of 0.2 mg. ATP–P into right carotid artery through cannula in lingual artery (at A and B) and into left coronary (at C). Between A and B mass ligation of right occipital, internal carotid and other small arteries leading to brain, but leaving sinus nerve intact. Between B and C thorax opened; artificial ventilation; heparin; cannula tied into left coronary artery for perfusion. Time in seconds.

Inhibition of the sympathetic is not involved in the reflex slowing of the heart, since the removal of the stellate ganglia did not diminish the effect.

The mechanism of the reflex bradycardia appears to be similar to that in cats and originates, at least to some extent, from the heart itself. In a few experiments the left coronary artery was perfused and an injection of ATP into it produced bradycardia provided the vagi were not cut (Fig. 7 C).

Respiratory changes in cats

An intravenous injection of 0.1 to 0.4 mg. ATP – P produces profound changes in pulmonary ventilation. There is often immediate cessation of movements lasting for 10 to 50 seconds. The apnoea precedes the fall of arterial blood pressure or occurs simultaneously. In some experiments apnoea was absent ; instead there was a period of shallow frequent respiratory movements. Both changes might be the sole effects or they might be followed by strong hyperventilation with increased depth and frequency of breathing and sometimes incomplete expiration. Some of these changes are illustrated in Fig. 8: at A there is a period of apnoea, at D a period of shallow frequent breathing in cats under chloralose ; at B and C there are periods of apnoea followed by strong hyperventilation, which at C occurs with incomplete expiration ; these tracings are from decerebrate cats. Injections of creatine phosphate (0.6 mg. CrP – P), adenylic acid (10 mg.) or sodium pyrophosphate (10 mg.) do not produce similar changes in pulmonary ventilation.

A complete analysis of the respiratory changes has not been carried out. The results so far obtained indicate, however, that several mechanisms are involved. ATP has a direct action on the respiratory centre and, in addition, seems to affect the centre indirectly by reflexes originating in the lungs, the impulses being carried via the vagi. No experiments have been performed to find out if it affects the centre also via the chemoreceptors.

The initial period of apnoea is brought about, partly at least, by a reflex via the vagus ; partly it

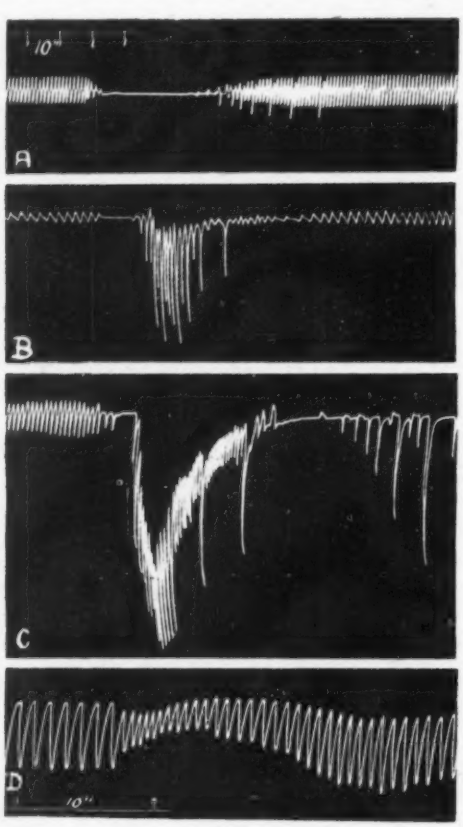


FIG. 8.—Record of respiration from four cats. A and D under chloralose, B and C decerebrate. Effect of intravenous injections of 0.1 mg. (in A and C) and 0.2 mg. (in B and D) of ATP–P. Time in 10 seconds shown on top of the figure for A, B and C and at the bottom for D.

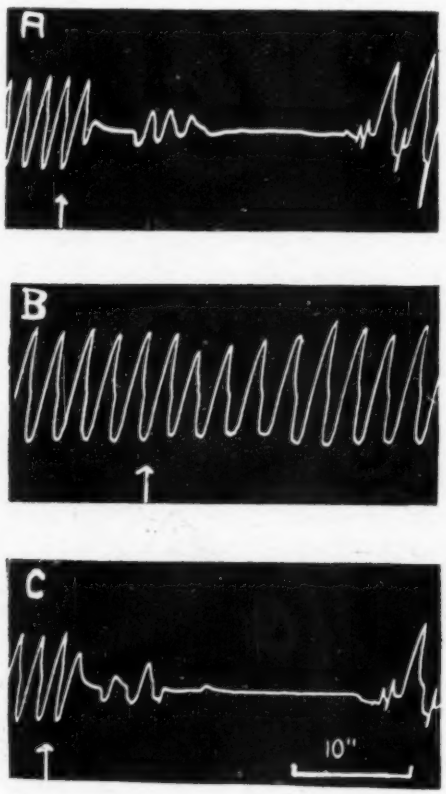


FIG. 9.—Record of respiration from 3 kg. cat under chloralose. At A, B and C intravenous injection of 0.4 mg. ATP–P . At B both vagi cooled at the neck. Time in 10 seconds.

may result from a central action of ATP. Cutting the vagi certainly reduces and sometimes even abolishes the apnoea. A certain caution, however, is necessary in the interpretation of this result, since repeated injections of ATP may cause reduced effects on respiration even without cutting the vagi. This argument does not apply to those experiments in which the vagi were cooled and the effect was found to be reversible. A striking experiment of this kind is illustrated in Fig. 9.

Some evidence in favour of the conception that the apnoea, as far as it is brought about reflexly, is due to stimulation of afferent fibres in the lungs, is given by an experiment in which the effect on respiration of 0.2 mg. ATP – P injected intravenously and into the ascending aorta has been compared. Instead of the initial apnoea produced by the intravenous injection there is a period of irregular breathing on arterial injection and, after cutting the vagi, the intravenous injection also produced these changes only.

A direct effect of ATP on the respiration centre is assumed from the fact that cutting the vagi usually did not wholly eliminate the respiratory changes and from the effects obtained when ATP was injected into a vertebral or carotid artery with denervated carotid sinus region. These injections produced respiratory changes, but they were weaker than those obtained with the same dose of ATP injected intravenously and with intact vagi.

Muscular contractions in cats

As mentioned earlier the experiments on circulation and respiration with ATP were the outcome of an incidental observation: i.e., widespread muscular contractions. These were obtained regularly in decerebrate cats on intravenous injection of 0.2 to 0.4 mg. ATP – P, but in cats under chloralose anaesthesia larger amounts of ATP had to be injected in order to produce this effect and even then it was not obtained regularly or in as pronounced a form as in decerebrate cats; only the latter therefore were used for further analysis.

The contractions consisted of a typical pattern. After a latency of between 15 to 20 seconds the forelegs became maximally extended and extreme opisthotonos developed. Sometimes the hind legs too became strongly extended. In several experiments, particularly when the cat was lying on its side with the head elevated, co-ordinated running movements occurred especially in the forelegs. Micturition, defaecation and strong peristalsis sometimes occurred. A similar pattern of muscular contractions to that observed after ATP could be elicited in the decerebrate cat on occluding the vertebral arteries with arterial clips; this was followed within less than 10 seconds by rigid extension of the forelegs and strong opisthotonos. It appeared possible therefore that anaemia of the central nervous system, caused by the fall in arterial blood pressure or stoppage of respiration, was responsible for the ATP contractions. But the effect occurred also when the depressor action of ATP was greatly reduced by injecting the ATP into the left ventricle, after atropine and with artificial ventilation. Central anaemia may, however, be a contributory factor when ATP is injected intravenously. When injected into the left ventricle the muscular contractions start after a shorter latency than after intravenous injections. This observation excludes also a reflex action of ATP originating in the lungs as the cause for the muscular effects. They occur also when the vagi nerves have been cut. In two out of five cats, however, the dose of ATP necessary to produce the muscular contractions on intravenous injection had to be doubled after the vagi were cut; thus afferent impulses in the vagi may perhaps participate to some extent in the initiation of the muscular contractions. Essentially, however, they are the result of an action of ATP on the central nervous system and therefore easily obtained when ATP is injected into a vertebral artery; when this is done the latency is a few seconds only. In decerebrate cats an action of ATP on supraspinal levels is probably the main cause of the muscular contractions, but ATP has a stimulating action also on the cervical cord. When the spinal cord was transected just above or below the origin of the first cervical nerve roots the injection of 0.2 to 0.3 mg. ATP – P into a vertebral artery produced muscular contractions but of a pattern different from that seen in the decerebrate cat. The main effect was on the hind legs and resembled the pattern of a typical scratch reflex on one or both legs. Rapidly alternating flexion and extension occurred at the ankle, the knee and less at the hip. In fact some flexion at the hip was more or less maintained during these rhythmic movements. The effect usually started on the side where the ATP was injected and if weak the movements occurred only at the ankle or at the ankle and knee. When the effect was pronounced the feet of both hind legs were brought to the shoulder region and exerted here the typical scratching movements. The effect on the forelegs was less pronounced and consisted of fine clonic movements and extension. A similar pattern of muscular contractions was produced in these cats on clamping one or both vertebral arteries. The contractions produced in these cats, even those on the hind limbs, resulted from an action of ATP on the cervical cord, because no effect or slight muscu-

lar contractions only were observed when the same dose of ATP was injected into the descending aorta or into the central end of the superior mesenteric artery with the iliac arteries clamped during the injections. With larger doses (over 0.4 mg. ATP—P), these injections caused muscular contractions but after a latency of more than 30 seconds, i.e., after the ATP had passed the lower body circulation, the legs and the left heart. The strong congestion of the pulmonary artery and the right heart preceding these contractions was good evidence that the ATP had reached the pulmonary circulation in effective concentration.

Discussion

The symptomatology of ATP when injected intravenously into cats is a complex one including peripheral, reflex and central mechanisms.

The profound and steep fall in arterial blood pressure is accounted for to a great extent by obstruction in the pulmonary circulation leading to a diminution in cardiac output. Gaddum and Holtz (1933) have described the constrictor effect of ATP on the pulmonary arteries, but its decisive role for the depressor action of this substance has not been recognized. According to these authors other phosphate compounds show the effect to a smaller degree, and in other animals, at least in the dog, the pulmonary vessels are less sensitive to ATP than in the cat. It would be of interest to know if in the dog the smooth muscles of the hepatic veins show instead a special sensitivity to ATP. A second direct effect contributing to the depressor action of ATP in cats is the vasodilatation it produces in the systemic circulation. There is also the possibility, mentioned before but not yet examined, of vasodilatation brought about by a reflex and central action of ATP and inhibiting the sympathetic tone. A third factor which will accentuate the depressor action of ATP is a bradycardia sufficiently strong to cause reduced cardiac output. The bradycardia is to some extent only accounted for by a central action of ATP, the explanation given by McDowall and by Bielchowsky *et al.* because it is abolished by cutting the vagi. This procedure, however, does not exclude the possibility of a reflex action of ATP. We could in fact show that ATP stimulates afferent fibres of the vagus in the heart and thus reflexly produces bradycardia. The effect resembles that produced by veratrin alkaloids (Dawes, 1947). In cats under chloralose this reflex mechanism accounts for the whole effect, but in decerebrate cats in which the centre appears to be particularly sensitive to ATP and in dogs under chloralose anaesthesia central actions of ATP contribute to the bradycardia. According to McDowall and to Bielschowsky *et al.* ATP has in cats also a direct depressant effect on cardiac muscle, which is not abolished after section of the vagi. These authors, however, have not excluded the possibility that changes resembling those produced by a direct cardiac depression may easily be simulated by obstruction in the pulmonary circulation with its consequent engorgement of the right and insufficient blood supply to the left heart. In those experiments in which we injected ATP into the left heart and thereby avoided pulmonary obstruction no signs of depression of the heart muscle were seen. The position is different in rabbits in which depression of the cardiac muscle occurs with ATP and has been demonstrated in isolated perfused hearts.

The profound changes in respiration produced by ATP have, so far as we know, not been described previously. A more detailed analysis than we have performed would be necessary in order to evaluate the different mechanisms involved in this effect. As far, however, as the results go, they show that ATP affects the respiratory centre directly as well as reflexly, via impulses in the vagi probably originating in the lungs.

The muscular contractions observed on injections of ATP intravenously or into the left heart are central effects of ATP. They may be accentuated by the circulatory and respiratory changes causing central anaemia, because a similar pattern of muscular movements could be obtained on occluding the vertebral arteries. They may be further accentuated by afferent impulses in the vagi, because in two cats at least the dose of ATP had to be increased after vagotomy in order to elicit the muscular contractions. This might be explained, however, simply by the fact that the vagal slowing of the heart is one of the circulatory events which leads to the central anaemia.

Buchthal, Engback, Sten-Knudsen, and Thomasen (1947) were the first to describe centrally produced muscle contractions when ATP was injected into a vertebral artery of a chloralosed cat. They recorded action potentials from the muscles of the forelegs and attributed the effect to a stimulation of the anterior horn cells in the cervical cord, since in their experiments the vertebral arteries were occluded at the level of the atlas and injections of Indian ink had shown that only the cervical segments were stained. We feel unable in our experiments to localize or to confine the stimulating action of ATP to these cells. The pattern of muscular movements varied in decerebrate and in spinal cats, but was the same whether produced by ATP or by occlusion of the vertebral artery. In

decerebrate cats a postural pattern was obtained similar to that seen when the head in these animals is strongly dorsiflexed: i.e., extensor spasm of the forelegs and opisthotonos. Such a pattern of muscular contractions suggests the possibility of a stimulating action of ATP on supraspinal levels. In spinal cats with the section just below the medulla oblongata or even at its lower end the pattern of muscular movements produced by ATP as well as by occlusion of the vertebral arteries resembled the scratch reflex. If the blood supply of the vertebral artery in these animals were limited to the cervical cord, this would indicate a representation of this reflex pattern in the cervical cord. It is interesting to note in this connection that injections of ATP into the descending aorta produced no or scarcely any contractions of the skeletal muscles, suggesting a special susceptibility of the cervical region of the cord to ATP.

The fall in arterial blood pressure, the reflex slowing of the heart, the obstruction in the pulmonary circulation, the vasodilatation, the peristalsis, micturition, vomiting, defaecation, and the muscular contractions of spinal and supraspinal origin, all this complex symptomatology is one which cannot be reproduced by other phosphate compounds, although they may have one or another action in common with ATP. On the other hand this symptomatology is not confined to ATP. For instance a striking similarity is found, at least in cats, with the effects produced by serum or plasma. The toxic actions of serum in cats were first examined by Brodie (1900). His records of the changes occurring in blood pressure and respiration are undistinguishable from the tracings we obtained with ATP. In cats, serum or plasma also produce a pronounced bradycardia, which was shown by Brodie to be of reflex origin, the reflex according to his analysis being initiated in the lungs. A re-examination of the effect, however, has shown that the heart is to some extent at least the site from which the reflex originates (Dawes and Feldberg, 1948). Like ATP, serum produces in cats strong contraction of the pulmonary vessels and on its intravenous injection this action may lead to the disappearance of the heart oscillations on the arterial blood pressure tracing and is in fact considered to be the main cause of the fall in pressure (Reid and Bick, 1942). Gilding and Nutt (1944) have further observed, on injection of stored plasma into cats, peristalsis, micturition, vomiting, defaecation, and muscular contractions of a pattern similar to that seen with ATP. They obtained the contractions only in decerebrate and not in chloralosed cats, but we have seen that ATP also was much more effective in this respect in a decerebrate cat. According to Gilding and Nutt the contractions are abolished by cutting the vagi and would therefore be of reflex origin. The contractions produced by ATP are of central origin; however, in some experiments section of the vagi necessitated a doubling of the dose of ATP in order to elicit the effect, and if the dose had not been increased the impression might easily have been gained of a reflex nature of the muscular contractions. The experiments with serum thus need re-examination in the light of these findings.

One might be tempted from such a close similarity of action to assume that serum and stored plasma owe their action to the presence of ATP; this, however, is not so; otherwise the "toxicity" of serum and plasma would not be confined to cats because ATP exerts its actions also in other animals. Adenylic compounds have been considered as the cause of the toxic action of serum or plasma. According to Zipf (1930), adenylic acid is identical with the "Frühgift" of serum, but adenylic acid cannot produce the toxic effects in cats. All the evidence available suggests in fact that the principle which makes serum and plasma so toxic for cats is a non-dialysable substance and probably a protein of the albumin class (Brodie, 1900; Reid and Bick, 1942; Gilding and Nutt, 1944). There is no evidence to suggest that ATP is linked to a protein constituent in serum and that in this linkage it would be active in cats but not in other animals.

Summary

1. In cats under chloralose or in decerebrate cats the intravenous injection of 0.2 to 0.4 mg. ATP – P causes (*a*) a steep fall in arterial blood pressure due to constriction of the pulmonary vessels, bradycardia, and vasodilatation, and (*b*) profound changes in respiration. In decerebrate cats these injections, in addition, regularly produce muscular contractions and often peristalsis, defaecation, vomiting, and micturition. In cats under chloralose anaesthesia ATP produces these additional effects only when given in larger doses and even then not regularly. The complex symptomatology of ATP cannot, or can in parts only, be reproduced by other phosphates.

2. The effect of ATP on the pulmonary vessels may be so strong that only little blood can enter the left heart. There is consequently a great reduction in cardiac output, which is to a great extent responsible for the steep fall in arterial blood pressure and which may cause obliteration of the heart oscillations on the blood pressure tracings.

3. The strong bradycardia produced by ATP, which causes a further reduction in cardiac output, results mainly from stimulation of afferent vagus fibres in the heart. This reflex mechanism accounts wholly for the bradycardia obtained in cats under chloralose. In decerebrate cats as well as in dogs under chloralose a central action of ATP contributes to the bradycardia seen after intravenous injections of ATP. On the other hand in rabbits the bradycardia produced by ATP is a peripheral effect.

4. The changes in respiration produced by ATP in cats consist of an initial period of cessation of respiration or of shallow frequent respiration often followed by a period of hyperventilation. ATP affects the respiratory centre directly and indirectly through impulses carried via the vagi and probably originating in the lungs.

5. The muscular contractions obtained in decerebrate cats on intravenous injections of ATP are of central origin and therefore also obtained on injection into a vertebral artery. They may be accentuated, however, by afferent impulses in the vagi and by central anaemia as a result of the circulatory and respiratory effects. The muscular contractions consist in the main of extensor spasm of the forelegs and episthotonos. In spinal cats with the cervical cord intact, intravenous or intravertebral injections of ATP cause muscular contractions, particularly in the hind limbs, of a pattern resembling the scratch reflex.

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THE MAINTENANCE OF A FILARIAL INFECTION (*LITOMOSOIDES CARINII*) FOR CHEMOTHERAPEUTIC INVESTIGATIONS

BY

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The purpose of this paper is to describe the maintenance in the laboratory of a filarial infection (*Litomosoides carinii*) which is suitable for chemotherapeutic and other types of investigations. The strain is kept in cotton rats (*Sigmodon hispidus*) and with one interruption it has now been maintained in our department for 2 years, during which time over 600 rats have been infected as shown by the presence of microfilariae in their peripheral blood.

History

Cotton rats began to be used in the United States for laboratory experiments about 1940; they were employed particularly in the study of viruses and rickettsiae. During more recent years these animals have been bred on a large scale in laboratories both in America and in Britain. Most of the rats used in the earlier work were wild ones which had been trapped in certain parts of Florida and Texas. Many of these wild rats were found to contain a filarial worm, *Litomosoides carinii*, which had previously been described in these and various other hosts by Travassos (1919), Mazza (1928), Chandler (1931), Ochoterena and Caballero (1932), Vogel and Gabaldon (1932), Chitwood (1933), and Vaz (1934); these are reviewed by Vaz. Culbertson and Rose (1944) showed that these spontaneous infections in wild rats were very convenient for experiments on the chemotherapy of filariasis, and much work was carried out by these authors (summarized by Culbertson, 1947) and by many other investigators in America holding O.S.R.D. contracts. The interest thus excited led to a determined search for the arthropod vector. Eventually, Williams and Brown (1945) showed that the worm developed in the tropical rat mite *Liponyssus bacoti* Hirst and transmission to clean rats was demonstrated by these workers (1946) and by Scott and Cross (1946). Descriptions of the morphology and life cycle of this mite have been given by Hirst (1913, 1914), Holdaway (1926), Dove and Shelmire (1931, 1932), and others. At the end of 1945 Dr. R. W. Williams, Dr. J. A. Scott, and other investigators kindly told one of the authors (F.H.) their latest results and demonstrated their methods for handling cotton rats. Dr. Williams and Dr. Scott kindly provided infected rats and mites which were brought back to Britain in February, 1946. Without their generous co-operation this work could not have been started. Further supplies of mites were kindly provided at a later stage by Dr. Cuckler, of Minneapolis, and by Prof. R. M. Gordon, of Liverpool. The objective of the work here described has been the development and practice of transmission on a large scale so as to obtain a good supply of infected cotton rats for chemotherapeutic investigations; during its progress, reports on various aspects of small-scale transmission have been published by Williams (1946), Scott (1946, 1947), Scott, Stembridge, and Sisley (1947), Hawking and Burroughs (1946), Bertram, Unsworth, and Gordon (1946), and Bertram (1946, 1947).

Methods

The methods to be described were designed for transmitting *Litomosoides* to the maximum possible number of cotton rats with the minimum of labour. It is fairly easy to transmit this worm in the laboratory to a few animals at irregular intervals; but the maintenance of a constant supply of large numbers of infected animals is much more difficult, requiring constant care and attention to numerous small details. The chief difficulty is the prevention of foreign mites or insects entering the tanks which contain *Liponyssus* and rats, and destroying or overgrowing the *Liponyssus*. A second difficulty is encountered in the maintenance of proper conditions of humidity in the tanks, so that they are humid enough for the mites to

multiply but not so sodden with rat urine that the mites are drowned. The methods now employed have been subjected to repeated modification in the light of experience, and perhaps further improvements will still be found possible. Since success depends to such a large extent on the observance of small details, our present technique will be described rather minutely, after which the reasons for the various procedures will be discussed.

Description of Present Technique

The process may be divided into five stages:

(1) The breeding of *Liponyssus bacoti* in pure culture.

(2) Infection of the mites with *Litomosoides* from infected rats.

(3) Infection of clean rats from the infected mites.

(4) Maintenance of the rats while the worms mature.

(5) Examination of the infected rats.

(1) *Breeding of the mites in pure culture*

The pure cultures are kept in the insectary described by Hunt and Davey (1947), in which the temperature is kept at approximately 24–26° C. and the relative humidity at 75 to 85 per cent. This room is used primarily for the maintenance of adult mosquitoes, but it is convenient to use it also for breeding the pure cultures of mites as the atmospheric conditions are ideal, the cultures occupy little space and, furthermore, they are isolated from contamination by foreign mites which may occur in the room used for the next two stages.

The colonies of mites are maintained in small glass jars (1-lb. jam jars) measuring approximately 8 by 13 cm. Before use the jars are washed. Clean sawdust about 2 cm. deep is put in the bottom of each, and on top of this a small piece of clean white filter paper is placed. The top of the jar is covered with finest bolting-silk or parachute nylon, the covering being tied round the neck with string. The jars and contents are sterilized in the autoclave (preferably the drying autoclave) and subsequently left 1–2 days in a 37° C. incubator to become thoroughly dry. The string fastening the silk covering is now removed from each jar and replaced by a rubber band. Mites (adults only) are removed from existing cultures by means of a fine camel's-hair brush (previously cleaned by dipping for a few minutes in ether) and floated off on to the surface of some clean water to remove any adherent matter. They are then transferred, again by brush, to a sheet of filter paper on which they are examined with a lens in order to make sure they are really *L. bacoti*, and that they have no smaller mites crawling on them, as is often the case. After examination they are transferred to the filter paper inside the jam jars. A thin film of dimethylphthalate smeared round the lip of a jam jar will prevent the escape of mites when the silk covering is removed. The effects of this repellent do not last more than one day, however. Each day one, or if possible two, day-old mice are put into each jam jar; or a day-old rat can be inserted every second day. To prevent contamination of the culture with unwanted species of mites which may be carried on the mice, each animal is placed in ether for about 10 seconds and then dried thoroughly in the air before being put into the jar. The mouse should be held gently with forceps throughout this operation. Mites feeding on a baby rat are shown in Fig. 1. Dead mice are removed daily with forceps, adherent mites being shaken from them back into the jar. Starting with ten mites in one jar it is possible in this way to breed about five thousand mites in a month, of which half may be expected to reach maturity.

A quicker but less safe method of "seeding" individual jars with mites is to remove a mouse from a jar which is already crowded with mites and to put it into the new jar. As the mouse will have a number of mites feeding on it at any given time, it is thus possible to carry over as many as a hundred mites in a single operation. However, mites should always be handpicked into one or two jars in order to maintain the stock culture, since otherwise there is considerable danger of contamination with foreign mites.

(2) *Infection of the mites with* Litomosoides *from infected rats*

This is carried out in an animal room set aside for the purpose. The temperature is kept between 27° and 32° C.; it is never allowed to fall below 24° C., as this will prevent *L. bacoti* breeding at the optimal rate. The relative humidity is maintained at about 50 per cent or 80 per cent, according to circumstances (see below), by means of a bucket of water standing over a small electric fire; the position of the bucket relative to the fire is adjusted when required according to the reading of a hygrometer on the wall. This simple arrangement is quite satisfactory for the present purposes. The mites (and rats) are kept in rectangular tanks, measuring 54×50 cm. by 26 cm. high, and made of 20-gauge galvanized iron sheeting (Fig. 2). The joints are brazed and riveted and the top edges of the sides are rolled; 4 cm. below the top edges, on the outside of each tank, is a horizontal ledge 1.7 cm. wide with an outer wall 1.3 cm. high, forming a channel which is normally filled with waste engine-oil. A rectangular wooden frame, of 3.6 cm. depth and 5.2 cm. wide, fits the top of each tank; on the under side there is a groove 1 cm. deep to fit over the edge of the tank; the recess is packed lightly with cotton-wool, which is soaked with liquid paraffin. Fine bolting-silk or parachute nylon is stretched over each frame and stuck down with petroleum jelly, and further secured by nailed laths. The joints of the wooden frame are packed with a plastic mixture of paraffin wax and petroleum jelly. Some metal tanks

FIG. 1.—Showing mites (the small dark objects) feeding on the posterior part of a baby rat.

FIG. 2.—Metal tank with lid (inverted) used for maintaining the colonies of mites while in contact with infected rats. Note (1) the groove in the lid packed with white cotton-wool to seal the top of the tank, (2) the gutter filled with oil outside the tank, and (3) the rat cage standing on two pieces of wood, on blotting-paper, on sawdust.

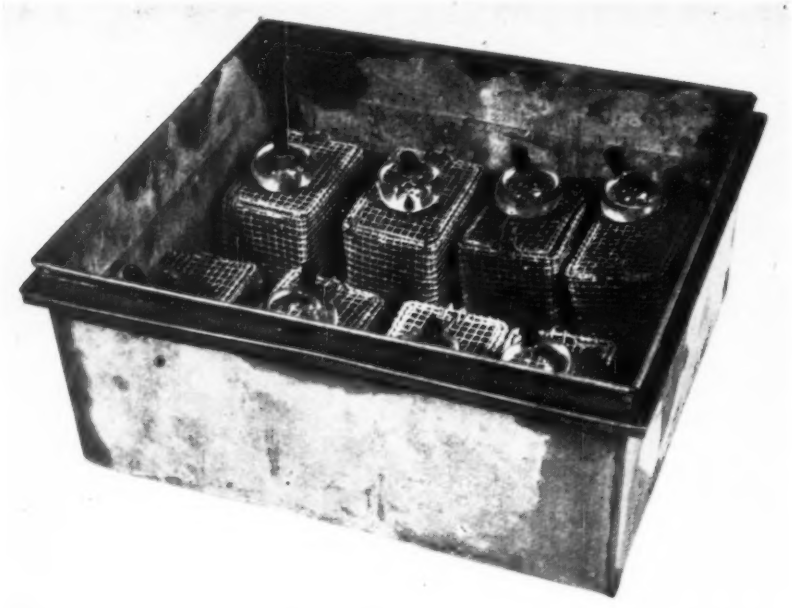

FIG. 3.—Metal tank containing eight clean rats in separate cages for exposure to infected mites; the glass sphere on top of each cage is a water bottle.

FIG. 4.—Room used for storage of rats while infection develops. The cages are stood in double rows on metal bars, with a tray of sawdust underneath to catch the droppings.

are also used of smaller dimensions, viz., 50 cm.× 40 cm. by 15 cm. high. The wooden lid of these is 10 cm. deep, so as to compensate for the shallowness of the tank. These second tanks do not have an external oil gutter. Consequently they must be stood on metal tables 125×60 cm. by 75 cm. high, which have an oil gutter 3 cm.×5.5 cm. deep round the margin, so as to prevent the escape of mites into the room. The tanks provided with an oil gutter are also stood on such isolated tables as a general rule, although they can also be stood on an ordinary table 75 cm. high. Wooden furniture is avoided as far as possible in this room, so as to avoid infestation with bed-bugs; if a wooden table is used, the legs are stood in small basins of oil or lysol.

Every effort is made to ensure that each unit (tank and lid) is proof against the entry of any small fauna —e.g., mites other than *L. bacoti*, lice, flies—which can prove dangerous to the life of *L. bacoti*. Nothing is put into the infection tanks which has not previously been sterilized or thoroughly cleaned. It is found useful to cover the hands and wrists with insect repellent (e.g., dimethylphthalate) before handling the tanks or their contents. Before use the tanks are steam sterilized. The lids are sprayed with xylene to kill any small fauna that may be present; they are placed on the tanks and allowed to dry. Sawdust sterilized in the drying autoclave is spread to a depth of about 5 cm. over the bottom of each tank. Several cotton rats infected with *L. carinii* and showing microfilariae in the peripheral blood are isolated in separate sterilized wire cages 15 cm. long × 10 × 10 cm. and sprayed with xylene from a scent-spray. (Great care is needed in order to avoid killing the rats with the xylene aerosol.) The cages are supported on clean metal rods resting on two opposite sides of a shallow tray containing dilute lysol, and the rats are left there until all trace of xylene fumes has disappeared. The cages, with enclosed rats, are then placed in the tanks containing the sawdust. Four thicknesses of dry sterilized blotting-paper are placed under each cage to catch excreta. The cage is raised about half a centimetre above the blotting-paper by being laid across two small sticks, so that accumulated faeces do not soil the bottom wires of the cage. The blotting-paper is changed every two days. Usually two infected rats are allotted to each tank, but more may become desirable if mites are very numerous. Mites are added to the tank by pouring into it the contents of one or more of the jars containing mite-colonies described in the previous section.

During their sojourn in the tanks the rats are kept on a minimum diet. Two to four grammes of cubed processed food, previously baked to sterilize the surface, and ten cubic centimetres of water per rat daily are sufficient. Particles of waste food must not be allowed to accumulate, and excess of urine must be avoided. Since the rats are serving to encourage breeding of the mites, as well as to infect them, they are left in the tanks for seven days, or until the mites are "swarming" (not only near the rats, but also over the whole of the sawdust), whichever time is the greater. (The mites show great activity if the observer breathes on to the sawdust.) The rats are then removed from the tanks and are "demited" passively by being left for a two-day period over the tray of lysol described above.

(3) *Infection of clean rats from infected mites*

The same tanks are used as in the previous section. Within three days of taking out the infected rats, two or three cages measuring 20×28 cm. by 10 cm. high are placed in the tank. Each cage contains five young female rats which have been freed from foreign mites by means of xylene as described above. Alternatively, eight of the small cages containing single rats may be inserted (Fig. 3). The blotting-paper is changed every day except Sunday to prevent the accumulation of urine and faeces. The soiled blotting-paper and the faeces lying on it usually contain many mites; in order to allow these to fall back on to the cage, the paper and its adherent faeces are stored for one or two days on top of the cages inside the tank before they are thrown away. The positions of the cages inside the tanks are transposed every day so as to compensate for any maldistribution of mites. The humidity of the room is adjusted according to the number of rats present. If few are in the tanks, the relative humidity is raised to 80 per cent; if many are present in the tanks, the humidity is allowed to fall to 50 per cent in order to encourage the drying of the blotting-paper and sawdust. A few days after the clean rats have been inserted, about 30–40 mites may be removed from the tank and dissected to look for worms. This confirms that the mites will become infective, otherwise the further procedure must be modified accordingly.

After fourteen days the clean rats are removed and demited as was described above. If the empty tanks seem well stocked with mites and free from contamination by foreign fauna, infected rats are inserted in order to infect the mites again, thus re-starting the whole cycle. Otherwise the contents of the tank are placed in an incinerator and the tank is cleaned and sterilized, after which it will be available for use as at the beginning.

(4) *Maintenance of the rats while the worms mature*

After the rats have been removed from the infection tanks and demited, they must be stored for about fifty days while the worms mature. During this waiting period, female rats can be kept in cages of five; males must be kept in separate small cages (15×10× 10 cm.). All cages have wire bottoms. They are arranged across the longitudinal bars of a big metal rack (Fig. 4). A few centimetres below each row of cages there are long metal trays to catch the excreta. The trays contain sawdust and are changed every two days. The rats are provided with an excess of the Institute cubed stock diet for rats and mice, supplemented by green cabbage three times weekly. Water is provided in excess in licking-bottles. The temperature of the room is maintained at approximately 25° C.

(5) *Examination of the infected rats*

Cotton rats are more difficult to handle than ordinary laboratory rats, since they are easily frightened and try to bite when gripped; also the skin on the tail is too fragile for them to be lifted up by this means. We have found that the most satisfactory technique is to approach the rat suddenly from behind and grasp it firmly by the scruff of the neck. A worker with sufficient confidence in himself can do this with the bare hand, without risk of injury; others will require stout leather gloves to give them a feeling of safety. An alternative technique is to take hold of the body by the hand protected by a leather glove so that the head projects between the thumb and forefinger. The rat twists round to bite the glove near the base of the thumb, but with a suitable glove there is so much slack leather at this point that the teeth do no harm. Meanwhile, blood can be easily taken from the tail or injections can be made into the posterior half of the animal. If there is difficulty in extracting a rat from a small cage, the rat is emptied out into a small dustbin more than 80 cm. deep—i.e., too deep for the rat to jump out. In this it can usually be grasped easily.

About eight weeks after removal from the infection tanks a drop of blood is taken from the tail of each rat and smeared on a slide to make a thick film, about 2 sq. cm. in area. These films can be stained by any of the usual techniques for microfilariae. We have found it rapid and convenient to dry them, to dehaemoglobinize in 1.5 per cent acetic acid, to dry again, and to stain with Leishman's stain.

After examination each rat is kept in a separate small cage. Rats which fail to show microfilariae three months after removal from the infection tanks are considered not to be infected, and they can be exposed to infection once again. We have no reason to believe that such rats are protected by a natural immunity, but the matter requires further investigation. If the number of infected mites in the tanks is high, there should be few failures to infect rats.

Comments on the Technique for Obtaining Rats Infected with *Litomosoides*

The previous section has described, somewhat dogmatically, the technique at present in use. Various features of this technique may now be discussed in more detail, to show the reasons for adopting them and our experience with alternative procedures.

(1) *Breeding of mites in pure culture*

The technique (described above) for breeding mites in jam jars has been in use for a year. It was started at a time when the mite population at our disposal was dangerously low, and the first jars contained soil and hay in imitation of the rat infection boxes then in use. Our whole present population is derived from three jars of one dozen mites each. We have not once had a jar contaminated with foreign mites. It is considered that this technique is more convenient and safer from contamination than the Single Unit technique described by Bertram, Unsworth, and Gordon (1946) or than methods requiring the isolation of mites after every feed.

Some observations on the habits of these mites are made possible by this technique. If the mites are well fed, with an excess of hosts (mice) present, they will not be seen above the sawdust except when actually feeding. Examination of the sawdust with a lens will reveal numerous stationary adults and highly active unfed protonymphs making their way upwards. Where the mites have insufficient food, however, they run rapidly over the inside of the jar, and will congregate on the inner side of the silk covering if there are mammals in the room. As soon as a mouse is put into the jar, further large numbers of mites will emerge from the sawdust and run rapidly about in the vicinity of the mouse. They appear to be under the influence of a simple chemotaxis which causes them to move directly towards the source of stimulation, until within one or two centimetres of it, when they will skirt about for some minutes before arriving at the source itself. Once on the mouse, young protonymphs will begin to feed almost at once. The older mites will run over the skin for as long as half an hour, before taking up a position to feed, usually at an orifice or in a fold of the skin. Behind the ears, in the groin, in the fold of skin between the shoulders, and around the mouth and nostrils are the favourite feeding places. The mites appear to stop running when they touch a feeding mite, and not infrequently start feeding themselves. In this way little clusters of mites soon form (Fig. 1). The older mites do not seem to interrupt their feeding as frequently as the protonymphs do.

(2) *Infection of the mites from infected rats*

As was said above, the maintenance of healthy colonies of mites during this and the next operation depends chiefly on the avoidance of contamination by other small fauna and the maintenance of appropriate humidity in the bedding. The tanks used were at first uncovered, but contamination with foreign mites persistently developed in spite of all precautions to prevent the accumulation of waste food and faeces. Even with the covers, most tanks become contaminated within six weeks of setting up. When contamination occurs, the

contents of such tanks should be sterilized as soon as the cycle of operations can be profitably terminated, since the population of *Liponyssus* usually diminishes rapidly, and the tank acts as a source of contamination for other tanks.

The commonest contaminants are mites of genera other than *Liponyssus*. When silk covers for the tanks were not used, the forage mite, *Tyrophagus castellanii* Hirst (and probably other similar species), was most frequently found, swarming over the fragments of food dropped by the rats, and on the licking-bottles. These mites had no appreciable effect on the *L. bacoti*. Less frequently a large yellow mite (adults 1.0 to 1.5 mm. long), *Macrocheles carinatus* Koch, would appear, and its appearance always heralded a decrease in the numbers of *L. bacoti*, so that we were led to the conclusion that this mite would eat either *L. bacoti* or its eggs. More recently a smaller yellow mite (adults 0.5 to 1.0 mm. long), *Hypoaspis freemani*, has been a frequent visitor, with similar disastrous consequences for *L. bacoti*. We have had little trouble from the Insecta. Lice have been seen once or twice, presumably feeding on the rats, but never in great numbers and never to the detriment of *L. bacoti*. A Dipteran, *Coprophila vagans* Hal. (a small black fly superficially resembling *Drosophila*), troubled us before the silk covers were introduced. It bred among the rat faeces and probably carried other contaminants from one tank to another.

The blotting paper underneath the cages was introduced in order to catch the urine, faeces, and waste food from the rats and to reduce soiling of the bedding. Pieces of cardboard were first employed and were moderately satisfactory; but they were difficult to clean and too expensive to renew every two days. Metal plates were tried but they collected pools of urine, in which the mites drowned, so they were quite unsuitable. Initially the waste material from the cages matted together in the meshes of the wire floor and formed an impervious sheet; this was obviated by inserting a small wooden bar, about 0.5 cm. thick, between the blotting paper and each end of the cage. The blotting paper should not project more than about 1 cm. round each cage, lest it hinder passage of the mites between the cage and the underlying sawdust. Young rats tend to gnaw the paper through the floor of the cage, reducing the upper surface to a powdery mass; this does little harm, apart from the mechanical inconvenience. The supply of drinking water to the rats has to be adjusted so as to prevent the blotting paper becoming soaked with excess of urine—otherwise conditions become unfavourable for the mites. The amount of urine which can be tolerated depends on the humidity of the atmosphere, on the number and size of rats in the cage, and on the frequency of changing the paper.

The bedding used in these cages originally consisted of a layer of dry sterile earth covered with sterile hay or straw as described by Williams (1946). Sawdust was found to give equally satisfactory results, with the additional advantages of cleanliness and the provision of a good background against which mites could be easily seen. The rats used to infect the mites should contain numerous microfilariae: the blood of our donors usually contains more than 50 microfilariae per cu.mm. If the mites are too numerous they may cause death of the rats by exsanguination; the danger-limit can be recognized only by experience. When it is approached, the donor rats can be changed, more donor rats can be added, or as a last resort the number of mites may be reduced by judicious spraying with xylene.

The percentage of mites which develop filarial infection during our procedure is small. Sample dissections have been done at various periods after exposing rats to the mites for 7–14 days. In 716 mites dissected within 6 days of the last day of exposure, 4.3 per cent were infected; of 419 mites dissected between the 7th and 13th days after exposure 1.4 per cent were infected, while of 240 mites dissected 14 or more days after exposure, none were found infected. For various reasons, these percentages are probably lower than the actual rates among the mites.

The length of the worms found in these mites varied from 60 μ to 800 μ, according to the period which had elapsed since the worm was ingested by the mite. This infection rate of the mites is much lower than has been reported by some observers—e.g., Bertram (1947) speaks of infection rates of 38 per cent. In our colonies the mites which have fed on the infected rats are constantly being diluted by young mites which have not so fed. All the same, there are sufficient infected mites in the colonies to transmit the parasite readily, and even in tanks where 50 or 100 mites were dissected without finding worms 50–100 per cent of the exposed rats became infected.

(3) *Infection of clean rats from infected mites*

The general problems of this stage are the same as those of the preceding stage. Greater care is required to prevent the blotting paper and bedding getting too wet. The caging of the cotton rats during this and the subsequent stage depends on their age and sex. The rats are most conveniently kept in groups of five, since each rat has more

freedom and the whole group occupies less space than five separate small cages. Unfortunately, confined groups of adult male rats or of mixed sexes are usually decimated by savage fighting; so that when pubescent or mature males are used they must be kept in separate small cages (Fig. 3). Restriction of use to female rats is convenient for handling, but it is rather wasteful of the breeding facilities.

(4) *Maintenance of rats while the worms mature*

The chief difficulties encountered during this phase arise from the number of rats involved and the problem of intercurrent mortality. If two dozen rats are removed from the infection tanks weekly and each must be stored eight weeks, accommodation is required for about two hundred rats, many of them in single small cages. The arrangement described (Fig. 4) is the most compact, most convenient, and most economical of attendant labour that we have yet been able to devise. At various periods there have been many deaths among our rats, sometimes averaging two or three per day, so that a quarter of our rats were being lost in this way before the infection matured. The causes of these deaths could not be satisfactorily determined. The distribution on the animal racks was not suggestive of contagious spread. Post-mortem examination of dead rats showed reddened intestines in some, thrombosis in the left auricular appendage of the heart in others, while others showed a clear effusion in the pleural cavities; intranasal instillation of this effusion into two other rats caused no obvious symptoms. Histological sections from a few of the rats suggested an infection with *S. typhi-murium*. Feeding experiments showed that growth was improved by adding green food to the diet, but otherwise the stock cubed diet for rats of the Institute seemed adequate. It was concluded that such sporadic deaths were best combated by attention to the general hygiene and diet.

(5) *Checking the rats for the presence of microfilariae in the peripheral blood*

Microfilariae do not appear in the tail blood of infected rats until 42 days after their removal from the infection tanks. (We habitually examine the rats for the first time after this period.) This means a possible maximum of 56 days from the first exposure to infection, and agrees with the conclusions of other workers that 51–54 days is the time required for microfilariae to appear. With light infections the appearance of microfilariae may be delayed until the 55th day after removal from the infection tanks. This may mean that the rats were not bitten by infected mites until their last day in the tanks, or that the number of microfilariae in the blood was too low to show in the first blood samples.

With most of our rats counts of microfilariae taken between the 40th and 60th days show a fairly consistent density of 10 to 15 per cubic millimetre of tail blood (about 2 per microscope field (× 60) of an average thick smear); thereafter the count increases to between 100 and 300 per cubic millimetre at the 100th day, and it remains nearly constant after that (so long as the adult worms are healthy).

Adult *L. Carinii* in the Host

We have never found adult worms in any part of the host other than the pleural cavity (and occasionally the mediastinum). However, we have occasionally failed to find worms in their normal location, although the blood of the host was positive for microfilariae, and so the possibility of successful development of the worms outside the thorax cannot be excluded.

Healthy worms in a healthy host tend to spread thinly over the wall of the pleural cavity, adhering by capillarity to the moist surface. They are seen to wriggle continuously in the dissected host; perhaps they are less active if undisturbed. If there is fluid in the cavity, the worms sink under the influence of gravity and become matted together. They also become matted if subjected to unfavourable conditions, e.g., if the host is treated with antifilarial drugs. When killed by drugs the mat of worms is soon covered with a fibrous deposit. In rats which have been infected for more than three months we have occasionally found spontaneous cures. Here, as in the drugged rats, we have found the worms matted together and covered with fibrin. In order to avoid mistaking such spontaneous cures for the results of drug action therapeutic tests are best performed with newly infected rats.

The worms are best examined in their living state. After removal from the pleural cavity the worms should be immersed in warm Ringer's solution and put in a 37° C. incubator for half an hour. At the end of this time normal worms show active motility, particularly in a bright light. They are nearly transparent, and most of their internal structure can be made out with care. We have noticed that after treatment with drugs of varying degrees of potency the behaviour and appearance of the worms are modified in a fairly constant fashion. The weakest effective drugs (or doses)

induce sluggishness, while stronger ones result in reduced transparency of the worms' tissues. When the worms are actually dying as a result of therapy they become covered with a thin layer of phagocytes, and later with fibrin, and they acquire a brown coloration. Even at this stage they may retain some motility. These changes are best seen a week after the course of injections has terminated, and they may be absent in rats killed earlier than this.

A moderate number of worms in the pleural cavity does not induce any gross pathological changes. If the number is excessive, however, the surface of the lung becomes inflamed and purulent. This may be due to penetration of the tissue by microfilariae.

Spontaneous death of the worms has never been found to occur in less than three months after removal of the rats from the infection tanks. When it does occur, the dead worms are usually found in a dense capsule of fibrin, and they exhibit little or none of their normal structure.

Transmission of Litomosoides *to animals other than cotton rats*

In the early descriptions of the worm now called *L. carinii* it was reported in various other small rodents, viz., *Sciurus*, and *Hesperomys* (Travassos, 1919), *Holochilus vulpinus* (Mazza, 1928), and *Nectomys squamipes* (Vaz, 1934). Accordingly, an investigation was made of the possibility of using other small rodents as experimental hosts for *Litomosoides* in place of the cotton rat. Animals were exposed to infection by the technique described above and the results are summarized in Table I. A preliminary note on these findings was published by Hawking and Burroughs (1946).

TABLE I

SUMMARY OF ATTEMPTS TO TRANSMIT *Litomosoides* TO ANIMALS OTHER THAN COTTON RATS

Species	Number of animals		
	Exposed	With worms in pleura	With Mf. in blood or pleura
Piebald rat ..	23	5	3
White mouse ..	70	11	2
Guinea-pig ..	2	0	0
Hamster .. .. (*Cricetus auratus*)	3	3	1
Orkney vole .. (*Microtus orcadensis*)	5	1	0

The piebald rats and mice which did become infected were consecutive ones out of a series spread over many months ; this suggests that if the others had been exposed to mites as heavily infected, they also would have developed worms. Some of the animals contained up to thirty worms, but most of them contained less than ten, both of which numbers are low for cotton rats. In piebald rats the microfilaria count in the peripheral blood rose to 1,000–5,000 per c.cm., and in two of the rats they persisted for more than two and three months respectively ; the rats were then killed. In all the animals of Table I the infections were much lighter than those usually found in cotton rats and there seemed to be a tendency towards spontaneous cure. It was concluded that although *L. carinii* will obviously develop in quite a variety of rodents (as shown by the literature and our own results) none of the species studied formed an efficient substitute for the cotton rat, which in fact remains the only satisfactory animal for chemotherapeutic experiments on this worm.

Chemotherapeutic Tests on Infected Rats

In designing a chemotherapeutic test consideration must be given to (1) allowing the drug sufficient opportunity to show its antifilarial activity (if any) ; (2) avoiding undue consumption of laboratory time, labour, and material. Culbertson and Rose (1944b) have shown that the effect of treatment of *L. carinii* infections with antimonial compounds must be judged by examination of the adult worms, since the microfilariae of the peripheral blood are not affected by doses which kill the adults. On the other hand, Hewitt and his colleagues (1947) have recently reported that piperazine derivatives act mainly on the microfilariae and only to a much smaller extent on the adult worms. Accordingly, examination of both adults and microfilariae is necessary in judging the antifilarial action of an unknown compound. Our preliminary experiments with neostam (Table II) suggested that treatment given on six successive days was more effective than treatment on three days only ; but treatment on twelve or eighteen days (which used much more time, labour, and drug) was not obviously more effective than treatment on six days. In view of these results and of reports by American workers, it was decided to utilize a dosage schedule of six daily doses. The procedure adopted is as follows :

When a compound is to be tested, a rough estimate is first made of its chronic toxicity for mice when given by intraperitoneal injection once daily for four successive days. Two mice are used per dose and each dose is approximately twice the

TABLE II

SPECIMEN RESULTS OBTAINED IN TESTING COMPOUNDS FOR FILARICIDAL ACTIVITY

Compound	Max. tolerated daily dose for mice (chronic toxicity) mg. per kg.	Treatment of rats				Condition of adult worms*
		Dose mg. per kg.	No. of doses	Interval (days)	No. of rats	
Neostam .. (stibamine glucoside)	250	40	6	1	2	Majority alive; a few dead
		40	18	1	2	All alive
		80	6	1	1	All alive
		80	12	1	2	All alive
		130	3	1	2	All alive
		130	3	2	2	All alive in one case; all dead in the other
		130	6	1	2	All dead in one; majority dead in the other
		130	12	1	2	All dead in one; majority dead in the other
		160	3	1	2	All dead
		250	6	1	1	All dead
		260	2	3	2	All dead in one case; all active in the other
		260	1	—	1	All dead
Pentostam .. (sodium stibogluconate)	2,500	200	6	1	1	Females injured; males unaffected
		500	6	1	1	All dead except a few of the males
		1,300	6	1	1	All dead
Anthiomaline ..	100	45	6	1	2	Males unaffected; females dead
Tryparsamide ..	2,500	250	6	1	2	Majority alive; a few females dead
		500	6	1	2	Males alive; females dead or dying
Neoarsphenamine	250	100	6	1	2	All dead
p-Methylsulphonyl benzamidine hydrochloride (V 187)	250	20–50	6	1	3	All alive

* Microfilariae were unaffected in all experiments

one below it. If the highest dose which has not killed any of the mice is x mg. per kg., the dose used for the therapeutic trial in cotton rats is $0.4x$ mg. per kg., given by intraperitoneal injection for six successive days; this dosage seldom proves toxic for the cotton rats. If the compound is found to be active, its toxicity can be investigated more closely. Infected rats are taken and a rough estimation is made of the number of microfilariae in the blood by counting the larvae in several typical microscope fields of a thick blood film. The rats are then treated by intraperitoneal injection given once daily on six successive days. Cotton rats infected with *L. carinii* do not tolerate the intraperitoneal injection of drug quite so well as healthy rats do. Also cotton rats are more susceptible than other laboratory animals to sepsis and bacterial infections during this procedure. All drugs should be dissolved in sterile fluid, and kept in sterile containers. Syringes and needles should be sterilized before use, and the site of the injection should be well cleaned with cotton-wool soaked in ether or in dilute alcoholic iodine solution before performing the injection.

Six days after the last dose the blood is examined again for microfilariae, the rat is killed, its thorax is opened, and the worms found in the pleural cavity are removed and examined. If their

motility is not obvious they are suspended in Ringer's solution and warmed to 37° C., which often stimulates them to motility. Often a mass of worms is found and it may be necessary to examine them under a microscope in order to estimate the proportion of living and dead ones. After effective filaricidal treatment, e.g., large doses of antimonials, the worms are usually found embedded in fibrin, forming small compact white masses. The microfilariae are much more resistant to treatment (by compounds other than piperazines) than the adult worms are, and when the adults have been killed by antimonials many active microfilariae can be found in the pleural fluid of the rat; living microfilariae also persist in the peripheral blood, where we have found them in gradually diminishing numbers for more than three months (as described by Culbertson and Rose, 1944b).

Examples of some of the therapeutic tests are reproduced in Table II. As reported by other observers, adult worms can be killed by suitable doses of antimonials (e.g., neostam, pentostam, or anthiomaline) and of arsenicals (e.g., trypars-amide). The males usually seem more resistant than the females. The microfilariae are unaffected by these types of compound. Most of the substances examined to date have unfortunately been inactive, e.g., V 187.

Discussion

In 1940 one of the authors (Hawking) described unsuccessful therapeutic investigations on patients with filariasis in East Africa, and pointed out that, although a chemotherapeutic remedy for filariasis would eventually be found, the testing of compounds directly on human beings was unlikely to produce the desired solution on account of the many difficulties and limitations inherent in such work; in order to be able to seek such a remedy with good prospects of success it was necessary first to obtain "an animal carrying a filarial infestation suitable for laboratory experiment; thus the animal must be small and cheap . . . and the problem of transmitting the infestation, presumably by insect vector, must also be solved." The infestation of cotton rats with *Litomosoides*, described above, seems to fulfil the conditions then laid down, and the prophecy then made that when this postulated experimental animal was available "the discovery of a potent filaricidal substance would be only a matter of time" is apparently in the process of fulfilment by the piperazine derivatives reported by Hewitt and his colleagues (1947).

The filarial infections hitherto available in the laboratory have consisted of parasites of dogs, lizards, and frogs. The filariae of dogs include *Dirofilaria inmitis* and *D. repens*; but dogs are too large and costly to keep in any quantity, naturally infected animals cannot easily be obtained in Britain (although they are available in the United States and Mediterranean areas), and transmission in the laboratory takes about ten months. Although dogs offer advantages for the study of the periodicity of microfilariae and for certain other studies on these stages of the worm, they are too big and expensive for the screening of new compounds. Filarial infections have been studied in lizards by Menon, Ramamurti, and Rao (1944), in frogs by Witenburg and Gerichter (1944), and in birds by many observers; but these infections are not easily available in Britain, transmission in the laboratory is difficult or impossible, and the metabolism of cold-blooded animals may be different from that of warm-blooded ones, so that experimentation in these hosts would be an unreliable guide to what might happen in man. Spontaneous infections of cotton rats with *Litomosoides* have already made possible investigations of the antifilarial action of antimonials (Culbertson, 1947, Culbertson and Pearce, 1946, and Culbertson and Rose, 1944a and b), of mercury and other compounds (Lawton *et al.*, 1945a and b), of the cyanine dyes (Welch *et al.*, 1947), and of the promising piperazine derivatives (Hewitt *et al.*, 1947; Santiago-Stephenson, Oliver-Gonzalez, and Hewitt, 1947). It is hoped that the technique described for transmitting the infection in the laboratory may render possible similar investigations in Britain and other countries where naturally infected rats are not available. Even where naturally infected rats are available, laboratory infections will probably prove preferable, being cheaper, more standardized, and more readily available in large numbers.

Summary

Large-scale methods are described for the breeding of *Liponyssus bacoti* and the transmission of *Litomosoides carinii* to cotton rats. During 1947 over 400 rats were infected by these methods in the authors' laboratory. These rats are used for screening compounds for antifilarial activity and the procedure employed is outlined.

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SUPPLEMENTARY NOTE

Since this text was written the following modification of technique has been introduced: Blocks of plaster-of-Paris 2.0–2.5 cm. thick are used to absorb urine and collect faeces in the infecting tanks; they take the place of the blotting-paper mentioned on p. 289. Each block is of such a size as to accommodate four small cages or one large cage with 1–2 cm. overlap. Faeces are periodically scraped off each block into a perforated zinc tray which hangs from the side of the tank; mites, which are usually plentiful in the faeces, can then return to the sawdust. Between periods of service in the tanks, which may be of up to two months' duration, the plaster blocks are steeped in water and then dried, when they are ready for use.

An important paper (Williams, R. W., 1948, *J. Parasit.*, **34**, 24) entitled "Studies on the life cycle of *Litomosoides carinii*" appeared early this year, too late to be discussed in the text.

Brit. J. Pharmacol. (1948), **3**, 297.

PHENYLTHIOURETHANES AS LOCAL ANAESTHETICS

BY

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(Received May 5, 1948)

Huang, Lu, and Chang (1946) found that the hydrochlorides of sym. bis(diethylamino)*iso*propyl phenylurethane and sym. dipiperidino-*iso*propyl phenylurethane exhibited marked local anaesthetic potency and relatively low toxicity. In view of these results and the similarity between the chemical behaviour of oxygen and sulphur, the hydrochlorides of two new phenylthiourethanes, viz. diethylaminoethyl phenylthiourethane (SD) and sym. dipiperidino-*iso*propyl phenylthiourethane (SPP), have been prepared and tested for toxicity and local anaesthetic activity. The formulae of these two compounds are given below.

(SD) $C_6H_5NH.CS.OCH_2CH_2N(C_2H_5)_2,HCl$

(SPP) $C_6H_5NH.CS.OCH(CH_2NC_5H_{10})_2,2HCl$

Experimental

Diethylaminoethyl phenylthiourethane hydrochloride was made by allowing sodium diethylaminoethoxide (prepared from diethylaminoethanol (2.35 g.) and powdered sodium (0.45 g.) in xylene (6 c.c.) on a steam-bath) to stand overnight with phenyl *iso*thiocyanate (3 g.). The product was acidified and the xylene removed in ether. The free base was an oil, but the hydrochloride crystallized from ethanol-ether; m.p. 121–122° C.; yield, 4.3 g. (Found: C, 54.0; H, 7.4; N, 10.0. $C_{13}H_{21}OSN_2Cl$ requires: C, 54.0; H, 7.3; N, 9.7 per cent.)

Sym. dipiperidino-isopropyl phenylthiourethane was prepared similarly from dipiperidino-*iso*propanol and phenyl *iso*thiocyanate. The free base crystallized from 90 per cent alcohol; m.p. 125–126° C. (Found: C, 66.6; H, 8.6; N, 11.7. $C_{20}H_{31}OSN_3$ requires: C, 66.5; H, 8.7; N, 11.6 per cent.) The hydrochloride crystallized when the free base was dissolved in warm 3*N* hydrochloric acid; needles, turning red at 200°, m.p. 235–236° C. (Found: N, 10.0. $C_{20}H_{33}OSN_3Cl_2$ requires: N, 9.7 per cent.)

Toxicity and anaesthetic potency

The experimental methods used were similar to those described previously (Huang *et al.*, 1946) except that guinea-pigs were used instead of human subjects for the intradermal weal tests. The results are collected in the Table. It will be seen that SD was about two-thirds as toxic as cocaine and SPP about equal in toxicity to cocaine. SD was about two-thirds and SPP about five times as potent as cocaine when tested on the rabbit cornea. When compared with procaine by the intradermal weal method, SD and SPP were about two and thirteen times as potent respectively.

TABLE

TOXICITIES AND ANAESTHETIC POTENCIES

Drug	LD50 in mice (Karber's method) mg./kg.	Anaesthetic potency (efficiency ratio, average of 7 experiments)	
		Rabbit's cornea	Intradermal weal (guinea-pig)
SD ..	165 (50)†	2/3	2
SPP ..	97 (50)†	5	13
Cocaine	102 (50)†	1	—
Procaine	—	—	1

† Total number of mice in parentheses.

The practical value of a local anaesthetic depends among other things upon its being non-irritant and stable to sterilization by heat. It was observed that both SD and SPP produced congestion of the conjunctiva in the rabbit's cornea tests and necrosis of the skin in the intradermal weal tests in concentrations at which local anaesthesia was achieved.

When solutions of SPP and SD were sterilized at 100° C. for one hour, the anaesthetic activity of the former was completely lost but that of the latter remained unchanged.

Summary

The hydrochlorides of diethylaminoethyl phenylthiourethane and sym. dipiperidino-*iso*propyl phenylthiourethane have been synthesized. Both exhibited marked local anaesthetic activity in the rabbit cornea and intradermal weal (guinea-pig) tests, but they are regarded as unsuitable for clinical application because they were found to be irritant. They had toxicities in mice of the same order as cocaine.

Reference

Huang, Y.-T., Lu, M.-C., and Chang, I. (1946). *Brit. J. Pharmacol.*, **1**, 273.

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Brit. J. Pharmacol. (1948), **3**, 298.

CURARE-LIKE ACTION OF POLYMETHYLENE BIS-QUATERNARY AMMONIUM SALTS

BY

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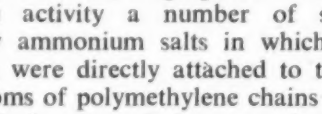
(Received May 11, 1948)

Tubocurarine chloride was shown by King (1935, 1936) to be a bis-tetrahydro*iso*quinolinium salt containing two quaternary nitrogen atoms. The curare-like blocking of neuromuscular transmission is a property of "onium cations" in general, and it was thought that the exceptional potency of tubocurarine compared with simple quaternary ammonium ions might be due in part to the presence of two such cationic groups at some optimal distance apart in the same molecule. With this in mind we have prepared and tested for curare-like activity a number of simple bis-quaternary ammonium salts in which the nitrogen atoms were directly attached to the terminal carbon atoms of polymethylene chains of different lengths. Such salts may be conveniently referred to as "bis-onium salts"; they were all polymethylene bis-onium dibromides of the general type:



Few compounds of this type have been examined previously; Brieger (1886) observed that tetramethylene bis-trimethylammonium hydroxide had some curare-like action in intact animals, and Ackermann (1921) made similar observations with the analogous ethylene and pentamethylene bis-trimethylammonium aurichlorides.

The following salts have been investigated.

1. Bis-trimethylammonium dibromides, $Br\{Me_3N(CH_2)_nNMe_3\}Br$, where n was 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, and 13. They are referred to as BTM followed by the number (n) of methylene groups in the chain; thus trimethylene bis-trimethylammonium dibromide is referred to as BTM3.

2. Bis-triethylammonium dibromides, $Br\{Et_3N(CH_2)_nNEt_3\}Br$, where n was 2, 3, 4, 5, 7, 8, 9, 10, and 13. They are referred to as BTEn.

3. Bis-strychninium dibromides, $Br\{C_{21}H_{22}O_2N_2(CH_2)_nC_{21}H_{22}O_2N_2\}Br$, where n was 2, 3, and 5. They are referred to as BSn.

4. Bis-quinolinium dibromides, $Br\{C_9H_7N(CH_2)_nC_9H_7N\}Br$, where n was 3, 5, and 10. They are referred to as BQn.

5. Bis-(phenyldimethylammonium) dibromides, $Br\{PhMe_2N(CH_2)_nNMe_2Ph\}Br$, where n was 3 and 5. They are referred to as BPDMn.

The following abbreviations will also be used: TM for tetramethylammonium iodide and TE for tetra-ethylammonium iodide

A brief note on some of the results with these bis-onium salts has already appeared (Barlow and Ing, 1948).

Method

The choice of a method for comparing the curare-like activity of numerous compounds is a matter of some difficulty; older methods have been reviewed by Ing (1936). Recently two methods have been devised for assaying tubocurarine solutions: Holaday (quoted by Bennett, 1941) developed the rabbit head-drop method and Chou (1947) used the isolated phrenic nerve-diaphragm preparation of the rat. We have tried to adapt Chou's method to the comparison of the curare-like activities of our bis-onium salts. Initially the apparatus and procedure were exactly the same as those used by Chou, except that the bath was smaller (50 ml. instead of 100 ml.). The condenser discharge stimuli used by Chou were of about 10σ duration and as there was a tendency for the nerve to discharge more than once they were later replaced by square wave stimuli of about 0.1σ duration. It was also observed during the course of the work that the diaphragm preparation is very sensitive to small changes of temperature and careful control of the bath temperature is necessary.

Chou allowed his tubocurarine chloride solutions to act for three minutes; we retained this feature of the method because if the drug is allowed to produce its full effect so much time is occupied by the poisoning

process and the eventual wash-out that few comparisons can be effected on one preparation. The disadvantage of this fixed-time method is that drugs may diffuse into the preparation at different rates. Ing and Wright (1931) were faced with the same difficulty in comparing the curare-like activities of tetra-alkylammonium salts on isolated nerve-muscle preparations of the frog, since they chose the time required for complete paralysis to indirect stimulation as their measure of curare-like activity; they were, however, able to show that diffusion rates were negligible with the nerve sartorius preparation, whereas they were far from negligible with the sciatic-gastrocnemius. The rat diaphragm is, like the frog sartorius, a thin muscle, and we thought that diffusion rates would probably be negligible; moreover, the latent period between addition of drug to the bath and diminution of the contractions was only a matter of seconds, whereas Ing and Wright found that the frog sartorius preparation exhibited latent periods of several minutes with onium salt solutions more dilute than 1 m*M*.

Our original intention was to use *d*-tubocurarine chloride as a standard and to compare all other bis-onium salts with it, but it was clear that we could only use an assay method of comparison if the dose-effect curves of bis-onium salts and *d*-tubocurarine chloride were parallel. The results of Bülbring and Chou (1947) indicated that the graph of percentage inhibition of the contractions against the logarithm of the dose was probably sigmoid, but when the probit of the percentage inhibition was plotted against the logarithm of the dose we found that the graph was approximately a straight line over the range of 10–50 per cent inhibition. We compared these regression lines for BTM10 and for *d*-tubocurarine chloride and found that the slopes and positions of the two varied widely and independently from preparation to preparation.

We thought it might be possible to obtain consistent results by comparing the doses of the two drugs required to produce a standard percentage inhibition. The ratios of the dose of *d*-tubocurarine chloride in µg. to the dose of BTM10 in ml. of 0.1 m*M* solution required to produce 16 per cent inhibition (probit = 4) on six separate occasions were: 5.1, 18.2, 4.8, 6.2, 18.5, and 7.6; the first four ratios were obtained with 95% O_2 + 5% CO_2 as the aerating gas, the last two with pure O_2. Clearly it was not possible to use *d*-tubocurarine chloride as a standard for comparison. The aerating gas was varied because Chou observed that the activity of tubocurarine chloride was less with pure oxygen than with an oxygen-carbon dioxide mixture (95% O_2 + 5% CO_2); we were unable to detect any such variation with our bis-onium salts.

In the method finally adopted one member (and usually the most active) of a homologous series was chosen as standard and as many as possible of the other members were compared with it on the same preparation, the aim being to find doses of all members which produced approximately the same percentage inhibition of the contractions in three minutes: in this way ratios of approximately equiactive doses were found, in terms of one member of the series, which revealed the way in which activities varied within the series. These ratios, the constancy of which on different preparations may be judged from Table I, were molar ratios, since all concentrations were expressed in mols or millimols per litre; no attempt was eventually made to relate them to some external standard, such as tubocurarine. The most active members of the BTM series were, however, compared with tubocurarine by means of the rabbit head-drop method with the generous help of Dr. N. K. Dutta.

Results

Bis-trimethylammonium (BTM) series.—The curare-like activities of members of this series varied in a rather unexpected way. BTM2 was about twice as active as TM; it might be considered simply to contain twice the number of effective blocking groups per molecule. The next three members of the series, BTM3, BTM4, and BTM5, were all very feebly active. BTM7 was about 1/6–1/7th as active as TM, BTM8 was about 50 per cent more active than TM, and BTM9 was 5 to 6 times as active as TM. BTM9, BTM10, BTM11, and BTM12 all had about the same order of activity and BTM13 appeared to be somewhat less active. The relative activities of the more active members of the series, as measured on several preparations, are recorded in Table I in terms of BTM9 = 1.

TABLE I

MOLAR RATIOS OF CURARE-LIKE ACTIVITY ON THE RAT DIAPHRAGM IN TERMS OF BTM9 = 1

Salt	Aeration with pure O_2			Aeration with O_2+CO_2		
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6
BTM2	0.30	0.25	—	0.30	0.5	—
,, 7	0.02	0.02	—	—	—	—
,, 8	0.25	0.20	—	0.25	0.3	—
,, 9	1.0	1.0	1.0	1.0	1.0	1.0
,, 10	0.6	0.8	0.6	0.6	1.3	—
,, 11	1.0	1.0	1.0	1.0	1.3	1.3
,, 12	0.6	0.8	0.6	0.6	—	—
,, 13	—	—	0.4	0.5	—	—
TM	0.15	0.13	—	0.13	0.2	0.2

Superficially these results suggest that members of the BTM series behave rather like the analogous alkyltrimethylammonium salts in which the alkyl group contains (to the nearest whole number) half the number of carbon atoms in the polymethylene chain; thus in the series $Me_3N.C_nH_{2n+1}$, the least active on frog muscle are those in which $n=2$ or 3 and cations in which $n=4$ to 8 are all about as

active as TM (Ing and Wright, 1931). In the BTM series the least active contain polymethylene chains of 3, 4, and 5 carbon atoms (the hexamethylene member was not prepared); members with polymethylene chains of 7 to 13 carbon atoms are all much more active. However, it is doubtful whether it is legitimate to compare activities on a rat preparation with those on frog preparations.

In the rabbit head-drop test the members of this series displayed entirely different orders of activity; as will be seen in Table II activity rose to a pronounced maximum for BTM10, which was some three times as potent, weight for weight, as *d*-tubocurarine chloride and about 10 times as active as the salts with 8, 11, 12, and 13 carbon atoms in the polymethylene chain. Only BTM9 approached BTM10 in activity, being about a third as active.

TABLE II

COMPARISON OF BTM SERIES WITH TUBOCURARINE CHLORIDE IN RABBIT HEAD-DROP TEST

Salt	Mean head-drop dose ± S.E. μg./kg.	No. of rabbits	Relative potency
Tubocurarine chloride	261 ± 14	5	1
BTM8	808 ± 52	2	0.3
„ 9	290 ± 27	4	0.9
„ 10	78 ± 20	6	3.3
„ 11	657 ± 107	2	0.4
„ 12	783 ± 81	2	0.3
„ 13	857 ± 43	2	0.3

Bis-triethylammonium (BTE) series.—The behaviour of members of this series had some resemblance to that of alkyltriethylammonium salts on frog muscle. Kulz (1922) recorded a steady increase of activity in the series $Et_3N.C_nH_{2n+1}$ from $n=2$ to $n=8$, and we have observed (on the rat diaphragm) a steady rise in activity from BTE4 to BTE13. The latter was about a hundred times as active as BTE4 and somewhat more active than BTM9. In the rabbit head-drop test BTE13, though the most active of the series on the rat diaphragm, was less active than BTM9 and about two-fifths as active as tubocurarine. BTE2 and BTE3 were only feebly active; BTE2 resembled TE in augmenting the response of the muscle to stimuli which had previously given maximal contractions (see below).

Bis-strychninium (BS) series.—The rat diaphragm is less sensitive to methylstrychninium salts than the frog's sartorius; thus, whereas Cowan and Ing (1934) found that methylstrychninium iodide was as active as tubocurarine chloride* on the isolated frog's sartorius (*R. esculenta*), we found that the chloride was not more active than TM on the rat diaphragm. The rat diaphragm is more sensitive to TM than the frog's sartorius: thus, 0.25 m*M* solutions of TM produced 50 per cent inhibition in 3 min., whereas Ing and Wright (1931, Fig. 1) found that 2.0 m*M* solutions were required to produce a similar inhibition of the isolated frog's sartorius in a similar time. At the same time TM is much less potent than tubocurarine on the rat diaphragm.

BS2 was more than twice as active as methylstrychninium chloride on the rat diaphragm (compare BTM2 and TM), but BS3 and BS5 were not significantly less active than BS2, a result which contrasts strongly with the feeble activities of the corresponding members of the BTM series. All bis-strychninium salts produced augmentation of the contractions of the rat diaphragm before inhibition.

Bis-quinolinium (BQ) series.—BQ3 and BQ5, unlike the corresponding members of the BTM series, show moderate activity. BQ10 was much less active than was expected; it was only about as active as BQ5, but it had a powerful augmenting effect on the muscle contractions, which must compete with the inhibitory effect.

Bis (phenyldimethylammonium) (BPDM) series.—Only two, BPDM3 and BPDM5, were prepared and tested, and, like the corresponding BQ compounds, they were both moderately active.

Augmentation effect.—It has already been noted that some bis-onium salts increased the size of the contractions of the rat diaphragm to maximal stimuli. With some salts, e.g., BTE2, this was the main effect and inhibition only occurred after a lapse of time much longer than 3 min.; with most salts the augmentation was only observed in some preparations. In the BTE series, apart from BTE2, which invariably showed it, the augmentor effect was observed from time to time with all the other members except BTE13; it was less frequently observed in the BTM series. Some preparations appeared to be much more sensitive to this augmentation (or possibly much less sensitive to the inhibitory effect) than others, and this variability in the response of preparations to these two opposing effects was probably the main reason for the difficulty in obtaining consistent estimates of relative activities; moreover, there was rarely a clear differentiation between concentrations which produced mainly augmentation and those

* The specimens used were King's crystalline *d*-tubocurarine chloride, at that time recently isolated, and Boehm's original amorphous alkaloid from tubocurare; they were wrongly referred to as "curarine chlorides."

which produced mainly inhibition. Three salts, BTE2, BS2, and BQ10, did, however, show such differentiation ; they caused augmentation at concentrations much lower than those required to produce inhibition. With BS2 it was possible to obtain, within the 3-min. interval, augmentation with low doses and inhibition with larger doses ; intermediate doses could also be found with which the two effects cancelled each other at the end of 3 min.

The cause of the augmentation is unknown ; its prominence with BTE2 recalls the similar effect of TE, which has been known for many years and appears to be due to a direct action upon the motor nerve (Cowan and Ing, 1933, 1935 ; Cowan and Walter, 1937). At the same time the augmentation resembles superficially the effect of anticholinesterase drugs and consequently representative bis-onium salts were tested as inhibitors of the cholinesterase of caudate nucleus (dog) with acetylcholine as substrate.

The following bis-onium salts were tested in this way: BTM9, BTE8, and BTE13 at concentrations which will inhibit the contractions of the rat diaphragm ; BTE2, BS2, and BQ10 at concentrations which will cause augmentation only. As will be seen in Table III, BTM9 had only slight inhibitory effect on the enzyme but all the other salts inhibited it markedly at these concentrations. TM ($2 \times 10^{-4}M$) had no inhibitory effect although augmentation was sometimes observed with this salt. BQ10 ($3 \times 10^{-5}M$) was also a powerful inhibitor of the cholinesterase of horse serum. This enzyme was also inhibited by BTE8 ($3 \times 10^{-4}M$) and by BTE13 ($3 \times 10^{-5}M$) but not by TM or TE ($3 \times 10^{-4}M$).

TABLE III

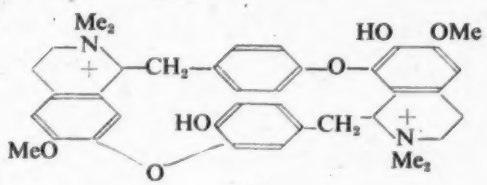
INHIBITION OF CHOLINESTERASE (CAUDATE NUCLEUS) WITH ACETYLCHOLINE ($6 \times 10^{-4}M$) AS SUBSTRATE

Salt	Molar conc.	Percentage inhibition
BTM9	3.7×10^{-5}	5.4
BTE8	3.3×10^{-4}	78.0
BTE13	3.7×10^{-5}	78.4
BTE2	3.7×10^{-3}	46.0
BS2	1.9×10^{-4}	30.5
BQ10	3.3×10^{-5}	93.0

Discussion

The main purpose of this work, which was begun in 1946, was to discover whether suitable separation of two quaternary ammonium groups in the same molecule would lead to enhancement of curare-like activity. The polymethylene bis-trimethylammonium series is the simplest possible type of bis-onium salt and it is of considerable interest that with a decamethylene chain a salt (BTM10) is obtained which is not only five to six times as active as TM on the rat diaphragm but also three times as potent as tubocurarine chloride in the rabbit head-drop test. Paton and Zaimis (1948) have independently confirmed the latter figure for the di-iodide corresponding to BTM10.

The occurrence of such high activity with a 10-carbon chain separating the two nitrogen atoms is particularly interesting. It may not be entirely fanciful to point out that in the tubocurarine structure I (King, 1948) the shortest route between the two nitrogen atoms traverses ten atoms (9 carbon, of which only one is extra-annular, and 1 oxygen) ; there are two such routes. The tubocurarine structure is not completely rigid, but the distance



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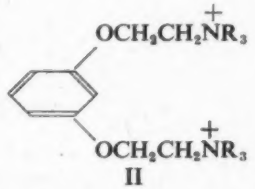

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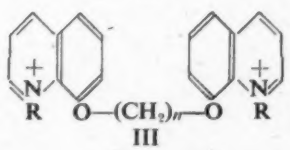

III

between the nitrogen atoms will vary little. Polymethylene bis-onium ions will be flexible structures capable of assuming a large number of configurations, subject only to the mutual repulsion of the two similarly charged nitrogen atoms. The decamethylene chain should allow distances between the two nitrogen atoms similar to those which occur in tubocurarine, and the same will probably be true for slightly shorter and for longer chains. If it be assumed that the exceptional potency of tubocurarine is due in part to the presence of two quaternary nitrogen atoms at some

optimal distance apart, we should expect high curare-like activity to occur with bis-onium ions capable of a similar separation of the two nitrogen atoms; such separation would probably involve a chain of at least 8 atoms. It is noteworthy that of the highly active bis-onium salts of types II and III studied by Bovet *et al.* (1946, 1947) the most active have 8 to 12 atoms between the two nitrogen atoms.

A reason may also be tentatively suggested for the occurrence of high activity in compounds containing *two* quaternary nitrogen atoms: if, in virtue of the distance between them, both charged centres are held by the "receptive substance," simultaneous dissociation of both centres from the receptors will be highly improbable and, so long as one centre is held, the re-attachment of the second is made more probable owing to the proximity of the ion to the receptive substance.

Our results with the BTM series indicate that the sensitivity of the rat diaphragm to bis-onium salts differs greatly from that of the rabbit. We also observed that the rat diaphragm was less sensitive to methylstrychninium and more sensitive to TM than the frog's sartorius (*R. esculenta*). Paton and Zaimis (1948) have noted that, compared with tubocurarine, BTM8 is some nine times as active in the cat as in the rabbit, and Brown and Dias (1948) have reported a striking difference in the sensitivities of *Leptodactylus ocellatus* and *Rana temporaria* to tubocurarine chloride. These differences in species sensitivity suggest that synthetic curare-like drugs ought to be tested on a variety of species.

The inhibition of cholinesterases by some of our bis-onium salts was unexpected and the specificity of this effect would probably repay further investigation. There is a rough correlation between the intensity of this effect and that of the augmentor effect in the rat diaphragm, but other possible explanations of the latter effect have not been explored.

Chemical Section

The bis-onium dibromides were prepared by heating polymethylene dibromides in ethanol with a large excess of the requisite tertiary base for from 5 to 24 hours. In the BTM series 33 per cent (w/v) aqueous trimethylamine was used; a pressure bottle was unnecessary.

Ethylene bis-onium dibromides were less easily prepared; BQ2 and BPDM2 could not be isolated. The product of heating ethylene dibromide with strychnine in ethanol is *bromoethyl-strychninium bromide* (Found: N, 5.62. $C_{23}H_{26}O_2N_2Br_2$ requires N, 5.38%). BS2 was prepared by heating this half-way product with excess strychnine at 180° C. for 7 hours in glycerol as solvent; the glycerol was removed, after acetylation, as triacetin by ether extraction; excess strychnine was extracted with chloroform; yield almost theoretical.

Condensations of polymethylene dibromides with quinoline and dimethylaniline give red and blue by-products respectively. The formation of red products with quinoline may be partly, and of blue products with dimethylaniline completely, suppressed by carrying out the reaction in an inert atmosphere (N_2 or CO_2); coloured products are best purified by pouring the reaction solution through a charcoal column.

Bis-onium dibromides are white crystalline solids. They are often both hydrated and hygroscopic; their analysis has presented some difficulty. Some salts could not be dried to a constant water content and the anhydrous salts were too hygroscopic for successful analysis; it was necessary to analyse the hydrated salt and use the experimentally determined water content in calculating the theoretical carbon and hydrogen figures. When an attempt was made to analyse some anhydrous salts, in particular BTE11 and BTE13, the specimens absorbed appreciable amounts of water between weighing and combustion; this can be detected by the high value for hydrogen and the low value for carbon, and if the excess hydrogen is assumed to be derived from water a "corrected" value for carbon can be obtained.

Analyses are by Drs. Weiler and Strauss. All m.p.s are uncorrected.

Ethylene bis-trimethylammonium dibromide. BTM2.
Crystallized from ethanol. No m.p. below 280°.
Found: C, 31.5; H, 7.9. $C_8H_{22}N_2Br_2$ required C, 31.4; H, 7.2%.

Trimethylene bis-trimethylammonium dibromide. BTM3.
Crystallized from ethanol. Loses water above 105° but does not melt below 250°. Roth (1881) does not record a m.p.
Found: C, 32.5; H, 7.87. Calculated for $C_9H_{24}N_2Br_2,1H_2O$: C, 32.0; H, 7.70%.

Tetramethylene bis-trimethylammonium dibromide. BTM4.
Crystallized from ethanol. Loses water below 110° but does not melt below 305°. v. Braun and Lemke (1922) recorded m.p. 295° (dec.).
Found: C, 32.7; H, 7.80. Calculated for $C_{10}H_{26}N_2Br_2,2H_2O$: C, 32.4; H. 8.11%.

Pentamethylene bis-trimethylammonium dibromide. BTM5.
Crystallized from ethanol. No m.p. below 280°.
Found: C, 37.2; H, 7.83; $C_{11}H_{28}N_2Br_2$ requires C, 37.6; H, 8.04%.

Heptamethylene bis-trimethylammonium dibromide. BTM7.
Crystallized from ethanol and ether. m.p. 113°.
Found: C, 38.3; H, 8.44; H_20, 7.86. $C_{13}H_{32}N_2Br_2$, $1.8H_2O$ requires C, 38.3; H, 8.70; H_2O 7.86%.

Octamethylene bis-trimethylammonium dibromide. BTM8.
Crystallized from ethanol. Loses water above 120° but does not melt below 280°.

Found : C, 40.0 ; H, 8.94 ; H_2O, 7.38. $C_{14}H_{34}N_2Br_2$, $1.7H_2O$ requires C, 39.9 ; H, 8.88 ; H_2O, 7.38%.

Nonamethylene bis-trimethylammonium dibromide. BTM9.

Crystallized from ethanol, acetone and ether. m.p. 101°.

Found : C, 41.3 ; H, 8.72 ; H_2O, 7.15. $C_{15}H_{36}N_2Br_2$, $1.7H_2O$ requires C, 41.3 ; H, 9.05 ; H_2O, 7.15%.

Decamethylene bis-trimethylammonium dibromide. BTM10.

Crystallized from ethanol. m.p. 113°.

Found : H_2O, 7.12. $C_{16}H_{38}N_2Br_2$,$2H_2O$ requires H_2O, 7.35. Sample dried at 100° in high vacuum. Found : C, 46.3 ; H, 9.28. $C_{16}H_{38}N_2Br_2$ requires C, 45.9 ; H, 9.10%.

Undecamethylene bis-trimethylammonium dibromide. BTM11.

Crystallized from ethanol. m.p. 104°.

Found : H_2O, 17.4. $C_{17}H_{40}N_2Br_2$,$5H_2O$ requires H_2O, 17.3. Sample dried at 100° in high vacuum. Found : C, 46.6 ; H, 9.44. $C_{17}H_{40}N_2Br_2$ requires C, 47.2 ; H, 9.26. "Corrected" value : C, 47.2%.

Dodecamethylene bis-trimethylammonium dibromide. BTM12.

Crystallized from ethanol and ether. No m.p. below 250°.

Found : C, 48.0 ; H, 9.17 ; H_2O, nil. $C_{18}H_{42}N_2Br_2$ requires C, 48.4 ; H, 9.42%.

Tridecamethylene bis-trimethylammonium dibromide. BTM13.

Crystallized from ethanol, acetone, and ether. No definite m.p.

Found : H_2O, 8.65. $C_{19}H_{44}N_2Br_2$,$2.5H_2O$ requires H_2O, 9.23. Sample dried at 100° in high vacuum. Found : C, 50.0 ; H, 9.63. $C_{19}H_{44}N_2Br_2$ requires C, 49.6 ; H, 9.57%.

Ethylene bis-triethylammonium dibromide. BTE2.

Crystallized from ethanol. m.p. 246°. Lucius (1907) recorded m.p. 245–246°.

Trimethylene bis-triethylammonium dibromide. BTE3.

Crystallized from ethanol. m.p. 264° (decomp.). Lucius (1907) recorded m.p. 245°.

Found : N, 7.06. Calculated for $C_{15}H_{36}N_2Br_2$: N, 6.93%.

Tetramethylene bis-triethylammonium dibromide. BTE4.

Crystallized from ethanol and ether. m.p. 273° (decomp.).

Found : C, 45.7 ; H, 8.83. $C_{16}H_{38}N_2Br_2$ requires C, 45.9 ; H, 9.09%.

Pentamethylene bis-triethylammonium dibromide. BTE5.

Crystallized from ethanol. No m.p. below 250°.

Found : N, 6.05. $C_{17}H_{40}N_2Br_2$ requires N, 6.48%.

Heptamethylene bis-triethylammonium dibromide. BTE7.

Crystallized from ethanol and ether. m.p. 274° (decomp.).

Found : C, 49.3 ; H, 9.35. $C_{19}H_{44}N_2Br_2$ requires C, 49.6 ; H, 9.57%.

Octamethylene bis-triethylammonium dibromide. BTE8.

Crystallized from ethanol, methylethyl ketone, ethyl acetate and ether. m.p. 240° (decomp.).

Found : H_2O, 16.17. $C_{20}H_{46}N_2Br_2$,$5H_2O$ requires H_2O, 15.95. Sample dried at 100° in high vacuum. Found : C, 50.1 ; H, 9.58. $C_{20}H_{46}N_2Br_2$ requires C, 50.6 ; H, 9.71%.

Nonamethylene bis-triethylammonium dibromide. BTE9.

Crystallized from ethanol, acetone and ether. m.p. 211°.

Found : C, 51.8 ; H, 9.62. $C_{21}H_{48}N_2Br_2$ requires C, 51.6 ; H, 9.84%.

Decamethylene bis-triethylammonium dibromide. BTE10.

Crystallized from ethanol, acetone and ether. m.p. 235°.

Found : C, 52.6 ; H, 9.91. $C_{22}H_{50}N_2Br_2$ requires C, 52.6 ; H, 9.99%.

Tridecamethylene bis-triethylammonium dibromide. BTE13.

Crystallized from ethanol, acetone and ether. m.p. 75–76°.

Found : H_2O, 15.48. $C_{25}H_{56}N_2Br_2$,$5.5H_2O$ requires H_2O, 15.5. Sample dried at 100° in high vacuum. Found: C, 54.1; H, 10.55. $C_{25}H_{56}N_2Br_2$ requires C, 55.1; H, 10.3. "Corrected" value : C, 55.0%.

Ethylene bis-strychninium dibromide. BS2.

Crystallized from ethanol. No m.p. below 300°.

Found : N, 6.76. $C_{44}H_{48}O_2N_4Br_2$ requires N, 6.55%.

Trimethylene bis-strychninium dibromide. BS3.

Crystallized from water ; saturated solutions contain about 3.2 g./1.

Found : N, 6.88. $C_{45}H_{50}O_2N_4Br_2$ requires N, 6.45%.

Pentamethylene bis-strychninium dibromide. BS5.

Crystallized from water ; saturated solutions contain about 1.6 g./1. No m.p. below 250°.

Found : N, 6.20. $C_{47}H_{54}O_2N_4Br_2$ requires N, 6.24%.

Trimethylene bis-quinolinium dibromide. BQ3.

Crystallized from ethanol. m.p. 247°.

Found: N, 6.35. $C_{21}H_{20}N_2Nr_2$ requires N, 6.10%.

Pentamethylene bis-quinolinium dibromide. BQ5.

Crystallized from ethanol. m.p. 195°.

Found : C, 53.6 ; H, 5.52. $C_{23}H_{24}N_2Br_2$,$2H_2O$ requires C, 53.1 ; H, 5.37%.

Decamethylene bis-quinolinium dibromide. BQ10.

Crystallized from ethanol and ether. m.p. 113°.

Found : H_2O, 5.88. $C_{28}H_{34}N_2Br_2$,$2H_2O$ requires H_2O, 6.06. Sample dried at 100° in high vacuum. Found : C, 60.3 ; H, 6.56. $C_{28}H_{34}N_2Br_2$ requires C, 60.2 ; H, 6.09.

Trimethylene bis-phenyldimethylammonium dibromide. BPDM3.

Crystallized from ethanol and ether. m.p. 213° (dec.).

Found : N, 6.30. $C_{19}H_{28}N_2Br_2$ requires N, 6.30%.

Pentamethylene bis-phenyldimethylammonium dibromide. BPDM5.

Crystallized from ethanol and ether. m.p. 226°.

Found : N, 5.52. $C_{21}H_{32}N_2Br_2$ requires N, 5.93%.

Summary

(1) The following series of polymethylene bis-quaternary ammonium dibromides have been prepared and tested for curare-like activity on the phrenic nerve-diaphragm preparation of the rat (n=number of carbon atoms in the polymethylene chain):—bis-trimethylammonium series, n=2, 3, 4, 5, 7, 8, 9, 10, 11, 12, and 13; bis-triethylammonium series, n=2, 3, 4, 5, 7, 8, 9, 10, and 13; bis-strychninium series, n=2, 3, and 5; bis-quinolinium series, n=3, 5, and 10; bis-(phenyldimethylammonium) series, n=3 and 5.

(2) In the bis-trimethyl series, the salt with n=2 is about twice as active as tetramethylammonium iodide; salts with n=3, 4, or 5 are only feebly active; activity increases from n=7 to n=9; salts with n=9, 10, 11, and 12 are all about 5–6 times as active as tetramethylammonium.

(3) In the bis-triethyl series, salts with n=2 or 3 are relatively inactive; activity increases from n=4 to n=13, the last member being somewhat more active than the bis-trimethyl member in which n=9.

(4) None of the members of the other three series was so active as the most active members of the bis-trimethyl series.

(5) In the rabbit head-drop test the bis-trimethyl member with n=9 was nearly as active as tubocurarine chloride; the member with n=10 was about three times as active. The bis-triethyl member with n=13 was about two-fifths as active as tubocurarine chloride.

(6) Some bis-onium salts, particularly bis-triethylammonium, bis-strychninium, and bis-quinolinium, augment the response of the rat diaphragm to maximal stimuli and inhibit the cholinesterase of caudate nucleus (dog).

Our thanks are due to Dr. N. K. Dutta, who carried out the rabbit head-drop tests.

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THE ANTIBACTERIAL ACTIVITY OF SOME SYNTHETIC COMPOUNDS RELATED TO PENICILLIN

BY

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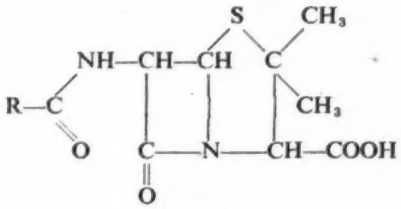
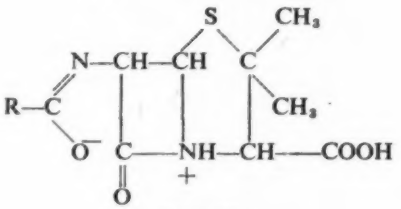
Concurrently with attempts to synthesize penicillin, the chemistry[1] and synthesis of which have been described by du Vigneaud, Carpenter, Holley, Livermore, and Rachele (1946), the antibacterial activity of compounds, or derivatives of compounds, known or postulated as parts of the penicillin molecule, has also been investigated.

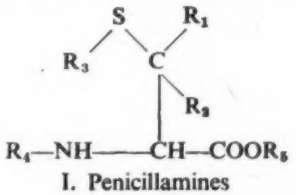
The compounds examined here may be conveniently classified into groups based on: penicillamines (I) (Copp and Wilkinson, 1947a ; Duffin and Wilkinson, 1947a, b ; Wilkinson 1947a) ; thiazolidine-4-carboxylic acids (II) (Wilkinson, 1947b) ; oxazolones (III) (Copp and Wilkinson, 1947b, c) ; derivatives of glycine (IV), and a miscellaneous group of intermediate and associated compounds (Copp, 1947) (included in Table II).



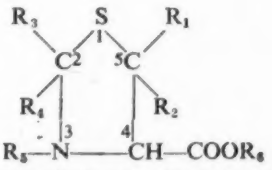
Penicillin
(with β-lactam structure)



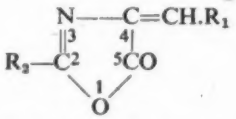
Penicillin
(with incipient azlactone structure)



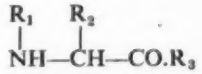
I. Penicillamines



II. Thiazolidine-4-carboxylic acids



III. Oxazolones



IV. Glycines

Results and Discussion

The antibacterial activity of the compounds, added aseptically to nutrient broth, was estimated by exposing falling concentrations of the compounds, in powers of two, to a constant inoculum of *Str. pyogenes*, CN.10. The results (Tables I and II) show that all compounds possess poor antibacterial activity *in vitro* when compared with penicillin (1,660 units per mg.). Those penicillamine esters (Table I) which possessed antibacterial activity were inactivated, partially or completely, by the presence of 10 per cent of blood or serum. The activity was not reversed by penicillinase and the morphological changes seen with penicillin were not observed.

The compounds marked T (Tables I and II) were examined for acute toxicity and for therapeutic activity in mice infected with *Str. pyogenes*, CN.10. All active compounds showed signs of toxicity within the range of 1–20 mg. per 20 g. mouse (0.5–1.0 g. per kg.), and no compound was chemotherapeutic. Loss of antibacterial activity of the esters in the presence of blood and serum suggested that the absence of chemotherapeutic action might possibly be due to hydrolysis. The

[1] Committee on Medical Research, O.S.R.D., Washington, and the Medical Research Council, London, *Nature*, 1945, **158**, 766, and *Science*, 1945, **102**, 627. The Editorial Board, Monograph on the Chemistry of Penicillin, *Science*, 1947, **105**, 653.

TABLE I

The antibacterial activity of a series of compounds expressed as units of penicillin per mg. The organism is *Streptococcus pyogenes*, CN.10. Penicillin contains 1,660 u/mg. Compounds marked T were examined for toxicity and chemotherapeutic action.

Compounds		*In vitro* activity expressed as units of penicillin per mg.			
		18 hrs.	48 hrs.	+10% blood	+10% serum
I. PENICILLAMINES					
S-Ethyl-cysteine		1/128	<1/128		
S-Ethyl-cysteine methyl ester	T	<1/128	<1/128		
Cystine *n*-butyl ester dihydrochloride		2	2		
dl-Penicillamine	T	<1/8	<1/8		
l-Penicillamine		<1/512	<1/256		
d-Penicillamine		<1/512	<1/256		
dl-Penicillamine hydrochloride	T	<1/8	<1/8		
dl-Penicillamine methyl ester hydrochloride		<1/8	<1/8		
dl-Penicillamine ethyl ester hydrochloride		1/8	1/8		
dl-Penicillamine *n*-propyl ester hydrochloride	T	1/4	1/4	1/8	1/32
dl-Penicillamine *iso*-propyl ester hydrochloride	T	1/4	1/4	1/8	1/16
dl-Penicillamine *n*-butyl ester hydrochloride	T	1/8	1/8		
dl-Penicillamine *iso*-butyl ester hydrochloride	T	1	1/4	1/4	1/8
dl-Penicillamine *n*-amyl ester hydrochloride	T	>8	16	1/4	1/8
dl-Penicillamine *iso*-amyl ester hydrochloride	T	>8	16	1/4	1/8
dl-Penicillamine *active*-amyl ester hydrochloride	T	>8	16	1/4	1/4
dl-Penicillamine *n*-hexyl ester hydrochloride	T	>8	8	1/4	1/4
dl-Penicillamine-*iso*-hexyl ester hydrochloride	T	>8	16	1/4	1/4
dl-Penicillamine benzyl ester hydrochloride	T	4	2		
N-Phenaceturyl-*dl*-penicillamine		<1/128	<1/128		
S-Benzyl-*dl*-penicillamine ethyl ester	T	1/4	2	1/28	<1/256
dl-Penicillaminic acid *n*-butyl ester hydrochloride	T	<1/128	<1/128		
N-Phenylacetyl-S-benzyl-*dl*-penicillamine methyl ester	T	<1/128	<1/128		
N-*n*-Caproyl-S-benzyl-*dl*-penicillamine methyl ester	T	<1/128	<1/128		
N-Formyl-*dl*-penicillamine methyl ester	T	<1/512	<1/512		
N-Formyl-S-benzyl-*dl*-penicillamine methyl ester	T	<1/512	<1/512		
N-Acetyl-penicillamine methyl ester	T	<1/512	<1/256		

TABLE II

The antibacterial activity of further groups of compounds against *Streptococcus pyogenes*, CN.10, expressed as units of penicillin per mg. Compounds marked T were examined for toxicity and chemotherapeutic action.

Compounds	*In vitro* activity expressed as units of penicillin per mg.		Compounds	*In vitro* activity expressed as units of penicillin per mg.	
	18 hrs.	48 hrs.		18 hrs.	48 hrs.
II. THIAZOLIDINES					
4-Carbomethoxy-2-phenyl-5 : 5-dimethyl-*dl*-thiazolidine hydrochloride T	1/32	1/64	4-Carboxy-N-benzoyl-2 : 2 : 5 : 5-tetramethyl-*dl*-thiazolidine (ammonium salt)	<1/8	<1/8
4-Carbomethoxy-2-aminomethyl 5 : 5-dimethyl-*dl*-thiazolidine hydrochloride	<1/8	<1/8	4-Carbomethoxy-2-*spiro-cyclo*hexyl-5 : 5-dimethyl *dl*-thiazolidine hydrochloride T	1/64	<1/32
4-Carboxy-N-phenylacetyl-2 : 2 : 5 : 5-tetramethyl-*dl*-thiazolidine (ammonium salt)	<1/8	<1/8	4-Carbo-*n*-amoxy-2-*spiro-cyclo*hexyl-5 : 5-dimethyl *dl*-thiazolidine hydrochloride T	1/4	<1/128

TABLE II—*continued.*

Compounds		*In vitro* activity expressed as units of penicillin per mg.	
		18 hrs.	48 hrs.
II. THIAZOLIDINES—*continued.*			
4-Carbomethoxy-2-carbethoxymethyl-2 : 5 : 5-trimethyl-*dl*-thiazolidine hydrochloride	T	1/32	<1/128
4-Carbomethoxy-N-benzoyl-2 : 2 : 5 : 5-tetramethyl-*dl*-thiazolidine	T	1/128	<1/128
4-Carboxy-N-formyl-2 : 2 : 5 : 5-tetramethyl-*dl*-thiazolidine		<1/8	<1/8
4-Amido-N-formyl-2 : 2 : 5 : 5-tetramethyl-*dl*-thiazolidine	T	<1/256	<1/256
n-Butoxy-N-formyl-2 : 2 : 5 : 5-tetramethyl-*dl*-thiazolidine	T	>1	1/2
4-Carbomethoxy-N-formyl-2 : 2 : 5 : 5-tetramethyl-*dl*-thiazolidine	T	<1/128	<1/128
4-Carbethoxy-N-formyl-2 : 2 : 5 : 5-tetramethyl-*dl*-thiazolidine	T	<1/128	<1/128
III. OXAZOLONES			
Δ²(4′-carboxy-5′ : 5′-dimethyl-thiazolidine)-2-phenyl-4-methyl-5-oxazolone		<1/128	<1/128
Δ²(4′-carbomethoxy-5 : 5′-dimethyl-thiazolidine)-2-benzyl-5-oxazolone	T	<1/128	<1/128
2-Phenyl-4-carbethoxymethyl-aminomethylene-5-oxazolone	T	<1/512	<1/256
2-Phenyl-4-benzylthiomethylene-5-oxazolone	T	<1/512	<1/256
2-Phenyl-4-(4 -aminobenzene-sulphonamido)-methylene-5-oxazolone		<1/128	<1/128
2-Phenyl-4-(2 -carboxyanilino)-methylene-5-oxazolone		<1/128	<1/128
2-Phenyl-4-(4′-aminoanilino)-methylene-5-oxazolone		<1/128	<1/128
2-Phenyl-4-(4 -amino-4 -diphenyl-aminomethylene)-5-oxazolone		<1/128	<1/128
2-Phenyl-4-(4 -carbethoxy-anilinomethylene)-5-oxazolone		<1/128	<1/128
2-Phenyl-4-(3′-aminoanilino-methylene)-5-oxazolone		<1/128	<1/128
2-Phenyl-4-(ethoxymethylene)-5-oxazolone		<1/8	<1/8
2-Phenyl-4-(ethylthiomethylene)-5-oxazolone	T	<1/64	<1/128
2-Phenyl-4-(benzylthiomethylene)-5-oxazolone	T	<1/512	<1/512
IV. GLYCINE ESTERS			
Glycine methyl ester hydrochloride	T	<1/32	<1/32
Glycine ethyl ester hydrochloride	T	<1/32	<1/32
Glycine *n*-propyl ester hydrochloride	T	<1/32	<1/32
Glycine *n*-butyl ester hydrochloride	T	<1/32	<1/32
Glycine *iso*-butyl ester hydrochloride	T	<1/32	<1/32
Glycine *n*-amyl ester hydrochloride	T	<1/32	<1/32
Triglycylglycine *n*-butyl ester	T	<1/32	<1/32
Phenylglycine	T	<1/512	<1/512
N-Formyl-glycine methyl ester	T	<1/512	<1/512
N-Formyl-glycinamide	T	<1/512	<1/512
α-Formyl-N-benzoyl-glycine ethylthioester	T	1/128	1/256
N-Benzoyl-α-ethylthiomethylene-glycinebenzylthio ester	T	<1/512	<1/512
N-Methyl-glycine ethyl ester hydrochloride (Sarcosine ethyl ester)	T	<1/512	<1/256
N-Benzylglycine ethyl ester hydrochloride	T	<1/512	<1/256
N′-(α-Formyl-N-benzoylglycyl)-*p*-aminobenzenesulphonamide	T	1/256	1/128
N′-(N-Benzoyl-α-ethylthio-methylene-glycyl)-*p*-aminobenzene-sulphonamide	T	1	1/8
N′(N-Benzoyl-α-benzylthiomethylene-glycyl)*p*-aminobenzenesulphon-amide	T	1/16	1/8
MISCELLANEOUS			
n-Butyl phenaceturate		1/128	<1/128
n-Amyl phenaceturate		1/64	1/128
Ethyl α-amino-ββ-dimethylacrylate	T	1/32	1/16
Methyl α-benzamido-ββ-dimethyl-acrylate	T	<1/32	<1/16
Ethyl α-(N-benzylbenzamido)-β-hydroxyacrylate	T	<1/512	<1/256
Ethyl α-(N-methylacetamido)-β-benzylaminoacrylate	T	1/512	1/512
Ethyl α-(N-methylbenzamido)-β-hydroxyacrylate	T	<1/512	<1/512
Mandelylalanine	T	<1/512	<1/512
Acetylmandelylalanine		<1/512	<1/512
Phenylaminoacetylalanine	T	<1/512	<1/512
N-Δ$^{\alpha}$-Hexenoylalanine (ammonium salt)	T	<1/512	<1/512
Hippurylamide	T	<1/512	<1/512
Ethyl-N-benzyl hippurate	T	1/512	<1/512
N-Benzylhippuric hydrazide	T	1/512	<1/512
5-Carbomethoxy-2-phenyl-tetrahydro-1 : 4-thiazone-3	T	1/32	1/16
5-Carboxy-2-phenyltetrahydro-1 : 4-thiazone-3 (ammonium salt)	T	<1/32	<1/16
bis-Phenylchloracetyl-cystine dimethyl ester	T	<1/32	<1/16
Benzyl mercaptan	T	<1/32	<1/16
Tribenzylthiocarbinol	T	<1/512	<1/512
2-Benzoylamino-3-pyrazolone	T	1/128	<1/128
Methyl *nor*penicillenate	T	<1/512	<1/512
Sodium *nor*penicillenate	T	<1/512	<1/512
3-Keto-4-carbethoxy-4-phenyl-Δ$^{\alpha}$-pentenoic acid	T	1/128	1/32
Formamide	T	<1/128	<1/128
dl-Valine *n*-butylester hydrochloride	T	1/128	1/256

observation that mice given toxic doses of *dl*-penicillamine-*n*-butyl ester hydrochloride by the intraperitoneal route were first anaesthetized and only later succumbed to the convulsions characteristic of penicillamine added verisimilitude to this possibility. Comparisons of the acute toxicity of *dl*-penicillamine-*n*-butyl ester hydrochloride and its constituents were made in groups of ten mice. The results (Table III) suggest that, when due allowance is made for the rate of hydrolysis, the acute toxicity of the compound approximates to that of the mixture of its constituent parts.

TABLE III

The average lethal doses of *dl*-penicillamine-*n*-butyl ester hydrochloride and its constituents when administered intraperitoneally to groups of 10 mice.

Compound	LD50 ± SD (mg. per 20 g.)
dl-Penicillamine hydrochloride	4.0 ± 0.6
dl-Penicillamine hydrochloride + *n*-butyl alcohol	10.5 ± 1.3
dl-Penicillamine-*n*-butyl ester hydrochloride	13.0 ± 1.6
n-Butyl alcohol	35.0 ± 5.7

Summary

1. Some penicillamine, thiazolidine, oxazolone, glycine, and associated compounds have been examined for chemotherapeutic activity.

2. The esters of penicillamine possess antibacterial activity *in vitro*, but their mode of action is not related to that of penicillin. They are inactivated in the presence of blood or serum, and evidence is presented which indicates that this may be due to hydrolysis.

3. The more active compounds, when administered by the intraperitoneal route, were acutely toxic in small doses to mice.

4. None of the compounds possesses chemotherapeutic value.

We have to thank Drs. F. C. Copp, W. M. Duffin, S. Smith, and S. Wilkinson of the Wellcome Chemical Research Laboratories for the compounds examined, Prof. A. H. Cook for the specimen of 2-benzyl △ $^{3}_{4}$-(4′-carbomethoxy 5′ : 5′-dimethylthiazolidine)-5-oxazolone, and Mr. M. W. Cheeseman for technical assistance in the investigations.

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STUDIES ON THE BLOOD-BRAIN BARRIER
I. THE BASIS OF DOSAGE FOR ANIMALS OF VARIOUS WEIGHTS

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According to common belief, the cerebral blood-vessels are more permeable in the young than in the adult animal. If any difference in permeability were such that the young blood-vessels allowed the passage of substances normally held back completely in older animals (e.g., many acid dyes), comparatively simple experiments would demonstrate it. If, however, the difference were merely one of degree, it would be apparent only if a satisfactory basis were reached for adjusting, to animals of very dissimilar body-weight, the dosage of a dyestuff or drug intended to pass these blood-vessels and reveal its presence in the nervous tissues by staining, by pharmacological effects, or by other means. None of our colleagues could state by what computation the adjustment should be made, and Clark (1937), discussing the dosage of drugs in general, wrote " there is no satisfactory method of relating dosage to body-weight when the latter varies extensively." The purpose of the present work was to inquire whether any general rule of dosing could be established for causing effects of equal intensity in the nervous tissues of animals of wholly dissimilar weight (and hence age).

Should dosage be related to the weight of the brain ? As Table I shows, the weight of the brain relative to the body-weight is enormously greater in the young than in the adult mouse. To a somewhat lesser extent, the same is true of the rabbit. Although, other things being equal, the concentration of drug in an organ is likely to depend on the concentration in the blood and not on the size of the organ, a consideration of the possible variables involved suggested that these were so numerous that it was advisable to settle the matter by direct experiment.

We had carried out preliminary experiments in which the dosage of dyestuffs and drugs was related to the body-weights or brain-weights of mice of different ages, or was calculated according to the metabolic-rate formula (metabolic rate $\propto$ body-weight $^{2/3}$). The results showed that computation of the dose according to brain-weight invariably led to much more severe effects in young animals. Between the other computations, the results did not permit of final choice ; of 7 dyes and 5 convulsant drugs, some appeared to affect mice of different ages more uniformly when administered on the basis of body-weight, others when given according to the metabolic-rate formula.

In the experiments described in the present paper, we adopted statistical methods of assess-

TABLE I

BODY-WEIGHTS AND BRAIN-WEIGHTS IN MICE AT DIFFERENT AGES

	Number examined	Average body-weight	Limits	Average brain-weight*	Limits	Brain-weight as % of body-weight
Old mice	90	41.0 g.	35.5–45.0 g.	0.44 g.	0.41–0.47 g.	1.1
Young adult mice ..	174	20.0 g.	19.0–21.0 g.	0.42 g.	0.40–0.42 g.	2.1
Three-week-old mice ..	174	11.8 g.	10.0–14.0 g.	0.40 g.	0.39–0.42 g.	3.4
Two-week-old mice ..	84	5.6 g.	not recorded	0.36 g.	not recorded	6.4

* Excluding olfactory bulbs and spinal cord.

ment. Because of the very variable response afforded by different animals, and of subjective errors in assessing results, dyes seemed unsuitable for the purpose. We used, therefore, three convulsant drugs (cocaine, strychnine, and picrotoxin) and compared the dosage-mortality curves for mice of three different age-groups; we chose these particular convulsants partly because they are believed to act at quite different levels of the central nervous system, and partly because they have been used previously in experimental work on epilepsy and the permeability of the blood-brain barrier (Cobb, Cohen, and Ney, 1938; Aird, 1939; Aird and Strait, 1944). In other tests we estimated chemically the amount of sulphanilamide passing into the brains of similar animals.

METHODS

Mice were selected according to weight. The weight of a mouse is a fairly precise indication of its age only if conditions of maintenance have been standardized and, at any rate in younger mice, the sizes of the litters have been reasonably constant. For the present purpose we used only mice from litters of two or three animals. These were raised from the age of two weeks under conditions as nearly standardized as possible with the prevailing standards of labour carefully supervised. Under these circumstances, the relation between weight and age was approximately as follows:

12 g.—2½ weeks; 20 g.—5–6 weeks; 35 g.—4 months.

Before experiment, all mice were kept at a constant temperature (±5° F.) for at least three days. On the day of experiment we checked each cage for the presence of surplus food and water. The drugs were injected intravenously, for mice of 20 g. (±0.5 g.),* dissolved in 0.1 c.c. distilled water (0.2 c.c. with sulphanilamide) and introduced in exactly 10 sec. (20 sec. with sulphanilamide) timed with a stop-clock. For mice of the other weights used (12 g. ±0.5 g. and 35 g. ±1.0 g.) the volume of the inoculum was adjusted to the body-weight; the time occupied in injection remained the same.

* In this laboratory mice of 20 g. are used in most experiments and are taken as the standard when comparison between various age-groups is made.

TABLE II

MORTALITIES IN GROUPS OF 10 MICE INJECTED INTRAVENOUSLY WITH VARIOUS CONVULSANT DRUGS

For mice of 20 g. the drugs were dissolved in 0.1 c.c. distilled water and injected in exactly 10 sec. For mice of 12 g. and 35 g. the volume was adjusted to the body-weight; the time of injection remained the same.

Drug	12 g. mice					20 g. mice					35 g. mice				
	Dose μg.	Mortalities Expt. No.				Dose μg.	Mortalities Expt. No.				Dose μg.	Mortalities Expt. No.			
		1	2	3	4		1	2	3	4		1	2	3	4
Strychnine hydrochloride	4	0				7	0				12	0	0	0	0
	5	0	1	1	1	8	0	0	1	1	15	1	0	2	1
	6	3	5	3	3	9	1	0			18	1	2	2	2
	7	3	6	5	5	10	2	3	5	3	21	4	5	5	5
	8	7	8	7	7	11	5				24	10	9	9	9
	10		9	9	9	12		5	7	6					
						14		7	8	8					
						16			10	10					
Cocaine hydrochloride	150	0				200	0				300	2	1	1	
	200	0	0	0		300	0	0	1		400	2	3	3	
	250		2	2		400	2	2	2		500	4	5	5	
	300	5	5	4		500	5	6	5		600	7	6	6	
	350		6	6		600	9	9	9		700	7	8	8	
	400	9	8	8		700		10	10		900		10	10	
	450		10	10											
	500	10													
Picrotoxin	25		0	0		50		0	0		50	0	0	0	
	30	2	3	2		60	3	2	3		70	4	1	1	
	40	4	5	5		70	6	6	5		90		2	2	
	50	4	8	7		80	9	8	8		100	8			
	60	7	10	9		90	9	9	9		110		6	6	
	70	9	10	9		100	10		10		130	10	10	9	
											160	10			

TABLE III

Probit-log. dose relationships for mortalities in mice from convulsant drugs: LD50 values, average slopes, and their standard errors.

Drug	Weight of mice		
	12 g.	20 g.	35 g.
Strychnine slope	8.41 ± 1.23	9.56 ± 1.47	11.80 ± 1.68
LD50 in mg.	0.0069 ± 0.0002	0.0113 ± 0.0003	0.0203 ± 0.0005
Cocaine slope	9.62 ± 1.46	10.22 ± 1.58	5.38 ± 0.85
LD50 in mg.	0.314 ± 0.010	0.472 ± 0.016	0.508 ± 0.024
Picrotoxin slope	6.27 ± 0.90	12.00 ± 1.71	9.00 ± 1.30
LD50 in mg.	0.0419 ± 0.0016	0.0680 ± 0.0017	0.0891 ± 0.0039

In the experiments with convulsant drugs, groups each of 10 mice received doses selected to cover as nearly as possible the whole range of mortalities from 0 to 100 per cent, with at least three values between the limits. We repeated this procedure three or four times on different days, with any variations of dosage suggested by previous experiments.

In the experiments with sulphanilamide, groups each of 3 mice received the drug and were killed 20 min. later. The choice of the time of autopsy was determined by experiments to be reported later. We estimated the amount of sulphanilamide in plasma and brain by the method of Rose and Bevan (1944). The experiment was repeated at approximately weekly intervals.

Results

(a) Convulsant drugs

Table II presents the experimental data for the convulsant drugs used.

The usual method of graphical representation of such data is to express the percentage mortalities as probits and plot these against the logarithm of the dose (Finney, 1947). A statistical analysis of the data of Table II shows that on this scale the dosage-mortality curves for all drugs are linear. The slope of a particular line is thus a measure of the individual variation of the mice to the drug in question (Finney). Further analysis of the data shows that, for each age-group and each drug, the slope of the line does not differ significantly in repeat experiments, indicating that individual variation remained the same throughout the period of experiment—i.e., for at least several days and in more than one batch of mice. Finally, the LD50 values did not differ significantly from one experiment to another. This is not a necessary condition of a satisfactory experiment, but it

TABLE IV

LD50 values and their standard errors expressed in terms of dose per 20 g. mice (mg./mouse) on body-weight, metabolic-rate, and brain-weight formulae.

Basis of assessment of dose	Drug	Weight of mice		
		12 g.	20 g.	35 g.
Body-weight mg./20 g.	Strychnine	0.0115 ± 0.0003	0.0113 ± 0.0003	0.0116 ± 0.0003
	Cocaine	0.523 ± 0.017	0.472 ± 0.016	0.290 ± 0.014
	Picrotoxin	0.0698 ± 0.0027	0.0680 ± 0.0017	0.0509 ± 0.0022
Metabolic-rate formula	Strychnine	0.0097 ± 0.0003	0.0113 ± 0.0003	0.0139 ± 0.0003
	Cocaine	0.442 ± 0.014	0.472 ± 0.016	0.348 ± 0.016
	Picrotoxin	0.0590 ± 0.0022	0.0680 ± 0.0017	0.0610 ± 0.0027
Brain-weight	Strychnine	0.0072 ± 0.0002	0.0113 ± 0.0003	0.0194 ± 0.0005
	Cocaine	0.330 ± 0.011	0.472 ± 0.016	0.485 ± 0.023
	Picrotoxin	0.0440 ± 0.0017	0.0680 ± 0.0017	0.0850 ± 0.0037

This table is derived from Table III. Doses administered according to the metabolic-rate formula were in the following ratio: 20 g. mice 1.00, 35 g. mice 1.46, and 12 g. mice 0.71. The approximate mean brain-weights derived from Table I were respectively 0.42 g., 0.44 g., and 0.40 g. These values and the body-weights were used in calculating the above data. Thus for 12 g. mice the LD50 for cocaine is 0.314. Expressed as dose per 20 g. mice on body-weight basis, this becomes $0.314 \times 20/12 = 0.523$. Calculated on the metabolic-rate formula, the dose would be $0.314 \times 1/0.71 = 0.442$, etc.

enables all the information for each drug contained in the experiments to be combined and represented by the average LD50, the average slope, and their standard errors (Finney).

By doing so we obtained the figures shown in Table III.

Interesting observations can be made from the figures given for the slopes. With strychnine the slopes do not differ significantly for the three age-groups ; this implies that the three age-groups have the same degree of variability in their reactions to strychnine. With cocaine the old mice have a significantly lower slope than the other two age-groups, indicating that so far as their reaction to this drug is concerned the old mice vary amongst themselves more than do mice of the other age-groups. For picrotoxin the reverse is true ; the young mice vary more amongst themselves than do the other two age-groups. Thus, comparing young and old mice, one may be more variable than the other with respect to one drug and less variable with respect to another. The 20 g. mice show up best for uniformity because with none of the drugs tested is either of the other age-groups significantly less variable.

In Table IV the LD50 is expressed in terms of the three methods of assessing dosage.

For strychnine the LD50 values based on body-weight are almost identical for the three age-groups. For this drug the best basis for calculating the dose is clearly by body-weight. For the other two drugs, none of the bases is completely satisfactory. There is reasonable agreement with picrotoxin for metabolic rate, but the body-weight basis is better when comparing young and 20 g. mice. With cocaine the only reasonable agreement is for brain-weights when comparing old and normal, and for metabolic rate when comparing normal and young mice. For the other group in both instances alternative computation would result in gross error.

All these conclusions are based on the LD50. Since the slopes are not always the same for different age-groups, the above comparative results only apply for the LD50. Different comparative results would be obtained for other levels of mortality, but the conclusion will hold substantially for levels between 20 and 80 per cent.

(b) Sulphanilamide

Table V shows the concentrations of sulphanilamide in the brains of mice of different weights. Obviously, to some extent, the amount of drug in the brain must depend on that in the plasma, and

TABLE V

AMOUNTS OF SULPHANILAMIDE PRESENT IN THE BRAINS OF YOUNG AND OLD MICE INJECTED INTRAVENOUSLY

The first line for each dose refers to one experiment, the second to another, and the third to the last experiment.
Doses for 12 g. mice (1) 0.6 mg. (2) 1.3 mg. (3) 2.0 mg.
„ „ 20 g. „ (1) 1.0 mg. (2) 2.0 mg. (3) 3.0 mg.
„ „ 35 g. „ (1) 2.0 mg. (2) 3.5 mg. (3) 5.0 mg.
For 20 g. mice the above doses were contained in a volume of 0.2 c.c. injected in exactly 20 seconds. For mice of other weights the volume was adjusted to the body-weight, the time of injection remained the same.
Ratio = concentration in brain/concentration in plasma.
Amounts are given in mg. per 100 ml. plasma or 100 g. brain; each pair of entries represent the concentration of sulphanilamide in the plasma and brain of a single mouse.

Dose	Weight of mice								
	12 g.			20 g.			35 g.		
	Plasma	Brain	Ratio	Plasma	Brain	Ratio	Plasma	Brain	Ratio
1	3.54	3.41		3.98	3.03		4.56	2.92	
	3.75	3.10		4.17	2.97		4.75	3.47	
	3.77	3.75		3.15	2.47		4:54	3.35	
	Mean 3.69	3.42	0.93	3.77	2.82	0.75	4.62	3.25	0.70
2	6.27	4.91		5.61	4.83		8.47	5.62	
	5.53	4.92		8.38	5.91		8.20	5.74	
	8.14	5.10		7.37	5.43		6.84	6.16	
	Mean 6.65	4.98	0.74	7.12	5.39	0.76	7.84	5.84	0.74
3	11.88	9.86		14.07	11.67		12.43	8.14	
	15.32	10.88		13.98	8.06		10.84	7.66	
	12.91	9.45		10.92	7.77		11.44	7.69	
	Mean 13.37	10.06	0.75	12.99	9.17	0.71	11.57	7.83	0.68

the ratio between these two quantities would appear to be the more significant figure. These ratios are fairly constant except for the lowest dose in young mice. The last may well represent a testing error resulting in an abnormally high value for the concentration in the brain ; it is not, however, sufficiently different from the other figures to warrant rejection of the result. The geometric mean of all nine ratios is 0.75, which may be taken as the ratio, 20 minutes after dosing, for a wide range of doses and weight of mouse. The reason for taking the geometric and not the arithmetic mean will be apparent in the following analysis.

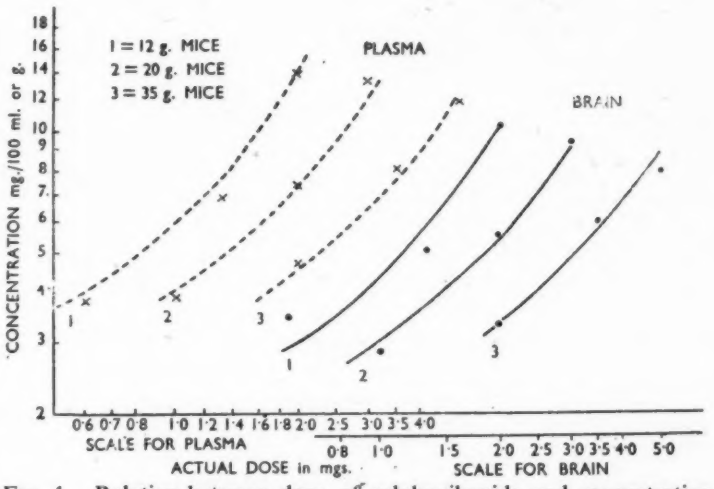

FIG. 1.—Relation between dose of sulphanilamide and concentration in plasma and brain for young, normal, and old mice.

Analysis of results

The mean concentrations in Table V have been plotted on log. scales in Fig. 1—i.e., log. concentration against log. dose. Some curvature is apparent in each graph, but it is clear that all curves are substantially parallel and that the relation between the plasma curves for young, normal, and old mice is similar to that for the brain curves. If the curves are in fact parallel and occupy relatively the same position within both sets (subject of course to experimental error), then the interpretation is greatly simplified. If the curves were not parallel, no uniform basis of calculating the dose would be possible, because the basis would then depend on the magnitude of the dose. If, for instance, the curves converged for low doses (and diverged for high), the ratios of the doses for young, normal, and old mice giving the same concentration of drug in the brain would be closer together for low than for high doses. The assumption that the curves are parallel was made, therefore, as a working hypothesis, to be subject to a rigorous statistical test at a later stage. If this assumption could be justified, a uniform basis for assessing doses would exist ; this basis would be the ratio of the doses which yield the same concentration of drug in the brain (or plasma) for all three age-groups.

The assumption is embodied by representing the six curves by the equations in Table VI.

Fitting these lines to the log. of the values of Table V by the method of Least Squares, we obtain the following values for the constants: $m_1=0.169$, $m_2=0.421$, $a=0.726$, $A=0.602$, $b=0.909$, $c=1.233$. These refer to the origin -0.222 for plasma and brain, and the theoretical curves are plotted in Fig. 1 ; it will be seen that the fit is reasonably good. The next step is to assess whether this fit is consistent with the experimental error. To do this we calculate for each animal the quantities

Y = log. concentration in plasma + log. concentration in brain.

Z = log. concentration in plasma − log. concentration in brain.

The variation within the groups of three mice for each dose and age group represents the experimental error for these quantities. An analysis of variance of Z shows that no significant variation arises between groups. It follows that the ratio of concentration in the brain to the concentration in the plasma may be assumed constant for all doses and age-groups of mice examined.

The fitted lines for the quantities Y for the three age-groups are derived by summing the corre-

TABLE VI

Sulphanilamide in the brains of mice: formulation of equations for log. concentration of sulphanilamide *v.* log. dose

	Plasma	Brain
Young	$y = a + bx + cx^2$	$y = A + bx + cx^2$
Normal	$y = a + b(x - m_1) + c(x - m_1)^2$	$y = A + b(x - m_1) + c(x - m_1)^2$
Old	$y = a + b(x - m_2) + c(x - m_2)^2$	$y = A + b(x - m_2) + c(x - m_2)^2$

sponding equations for the plasma and brain. The variance of the nine group means about these lines is 0.0065 based on 4 degrees of freedom. This has to be compared with the experimental error variance. We calculate that the variance of Y within groups of three mice is 0.0093 based on 18 degrees of freedom, accordingly the error variance of the group means is $0.0093/3=0.0031$. The variance about the fitted lines is not significantly greater than this. The fit of the lines is, therefore, adequate and the working assumptions justified.

We have now shown that within the experimental error, the ratio of the concentration in the brain and plasma is the same for all doses and weight of animal within the range examined, and also that the curves connecting the concentration in the plasma (and brain) with log. dose for the three age-groups are parallel.

The effect of age (or weight) of mouse on the concentration in the plasma and brain is therefore given entirely by the two constants m_1 and m_2 calculated previously—i.e., $m_1=0.169$, $m_2=0.421$. These are in terms of log. dose ; their antilogs. are 1.476 and 2.637. The relation between the doses to give the same concentration for young, normal, and old mice is, therefore, 1.00 to 1.476 to 2.637 respectively. Expressed as normal=100, we get 67 : 100 : 178.

In Table VII we compare these ratios with the three bases for assessing dose.

The ratios found agree almost entirely with the body-weight basis of assessment. The experimental error in the ratios found is unlikely ($p=0.05$) to exceed ± 12%. We can therefore confidently exclude the other two bases of assessment of the dose, since the experiment is on a sufficient scale to discriminate conclusively between the three bases and thus gives a clear-cut result. Fewer animals would have been insufficient and a larger number unnecessary. The conclusion is that the correct basis of assessment of dose is by body-weight.

TABLE VII

Sulphanilamide in the brains of mice: ratio of dose per mouse calculated in various ways compared with doses causing equal concentrations of the drug in the brain.

Basis of assessment of dose	Weight of mice		
	12 g.	20 g.	35 g.
Found experimentally ..	62	100	169
Body-weight	67	100	178
Brain-weight	95	100	105
Metabolic-rate formula ..	72	100	146

An estimate of the constant brain/plasma concentration may be obtained from a and A calculated above. The difference $A-a=1.876$ which corresponds to an antilog. of 0.75. This, of course, is identical with the geometric average determined previously from Table III, and is the justification for using the geometric in place of the arithmetic mean of the ratios.

Summary and Conclusions

The foregoing experiments sought to determine whether there exists any uniform basis of dosage, for mice of different weights, of drugs penetrating the blood-brain barrier. As an indication of their presence in the nervous tissue, we determined the mortality curves of three convulsant drugs, strychnine, cocaine, and picrotoxin. We also estimated chemically sulphanilamide passing into the brain. All the drugs were given intravenously under rigidly standardized conditions, thereby eliminating factors concerned with absorption from the alimentary canal or from the tissues.

Although, proportionately to the body-weight, the brain is relatively very much heavier in the young animal, dosage on the basis of brain-weight nearly always results in the administration of too large amounts of drug. Apart from this statement, no general rule can be formulated. With strychnine and sulphanilamide, dosage on the basis of body-weight gave results most nearly uniform in the various mice. With picrotoxin, dosage according to the metabolic-rate formula gave reasonably uniform results over the complete range of weights, but dosage on the basis of body-weight gave closer correlation between immature mice and young adults. With cocaine no basis of dosage appeared satisfactory for all weights. Computation according to the metabolic-rate formula yielded greatest uniformity between immature mice and young adults, according to the brain-weight between young adults and old mice.

In the absence of any uniform basis of dosage for mice of widely different weights, the recognition of small differences in the permeability of the cerebral blood-vessels of young and old animals would be a matter of considerable difficulty.

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THE DECARBOXYLATION OF β-3 : 4-DIHYDROXYPHENYLSERINE (NORADRENALINE CARBOXYLIC ACID)

BY

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β-3 : 4-Dihydroxyphenylserine is an amino-acid structurally closely related to noradrenaline. In this paper it will therefore be called "noradrenaline carboxylic acid."

The amino-acid was first prepared by Rosenmund and Dornsaft in 1919, and again by Guggenheim (1940), who found that in rabbits it was without action on the arterial blood pressure after subcutaneous injection of 0.1 g./kg. The acid has recently been prepared by Dalgleish and Mann (1947), together with the corresponding N-methyl-amino-acid, "adrenaline carboxylic acid."

Rosenmund and Dornsaft (1919) suggested that the amino-acid might be a precursor of adrenaline in mammals, but they did not support this suggestion by experiments.

A scheme of adrenaline synthesis has been discussed which assumes the decarboxylation of *L*-3 : 4-dihydroxyphenylalanine (Blaschko, 1939, 1942, 1948), a reaction catalysed by the enzyme dopa decarboxylase (Holtz, Heise, and Lüdtke, 1938). This enzyme does not act on N-methyl-3 : 4-dihydroxyphenylalanine (Blaschko, 1939), but it is not known how the introduction of a hydroxyl group in the β-position affects substrate specificity. We have therefore examined noradrenaline carboxylic acid as a possible substrate of mammalian decarboxylases.

The mammalian dopa decarboxylase is related to the bacterial *L*-tyrosine decarboxylase, and we have therefore included experiments on the decarboxylation of the amino-acid by the bacterial enzyme; these experiments have enabled us to establish the stereochemical configuration of 3 : 4-dihydroxyphenylserine.

Materials and Methods

For the noradrenaline carboxylic acid we are grateful to Dr. F. G. Mann, F.R.S. Two samples of noradrenaline hydrochloride were used for the biological assay; the first was a racemic preparation, known as Arterenol, which was obtained from the I.G. Farben-Industrie more than ten years ago; for the second sample, a specimen of the laevorotatory stereoisomer, we are grateful to Dr. M. L. Tainter.

The tissue extracts—from guinea-pig's kidney and suprarenal gland—were prepared as previously described (Blaschko, 1942). All our manometric experiments were conducted in an atmosphere of nitrogen.

An acetone-dried preparation was used for the study of the bacterial *L*-tyrosine decarboxylase. We are grateful to Prof. I. C. Gunsalus for a strain of *Streptococcus faecalis* R (ATCC 4083); the bacteria were grown on a medium similar to that described by Bellamy and Gunsalus (1945), but with an addition of 0.5 mg./litre of pyridoxal (Merck).

The pharmacological assay was carried out on the arterial blood pressure of the spinal cat; the rat's uterus preparation used in one experiment has been described by Garcia de Jalon, Bayo Bayo and Garcia de Jalon (1945).

Experiments

(1) *Experiments with guinea-pig's kidney extract*

The guinea-pig's kidney gives extracts of high dopa decarboxylase activity; we compared the rate of decarboxylation of the racemic 3 : 4-dihydroxyphenylserine with that of *DL*-3 : 4-dihydroxyphenylalanine. The manometric set-up was as follows:

	Flask 1	*Flask* 2	*Flask* 3
Main Flask	1.6 ml. guinea-pig kidney extract (in all flasks)		
Side Bulb	0.4 ml. water	0.4 ml. M/100 *DL*-3 : 4-dihydroxyphenylalanine	0.4 ml. M/100 noradrenaline carboxylic acid

Incubation temperature 37.5° C.

From one molecule of *DL*-dihydroxyphenylalanine half a molecule of carbon dioxide should be formed, as it is known that the decarboxylase is stereospecific and will form carbon dioxide only

from the *L*-isomer. The amount of carbon dioxide expected to be formed from 0.4 ml. of M/100 *DL*-amino-acid is 44.8 μl.

From 3:4-dihydroxyphenylalanine the theoretical amount of carbon dioxide was formed within the first nine minutes, and the reaction then came to a standstill. There was no formation of carbon dioxide from noradrenaline carboxylic acid.

(2) *Experiments with extracts from the guinea-pig's suprarenal gland*

There was no formation of carbon dioxide when extracts from the suprarenal gland were incubated with either 3:4-dihydroxyphenylalanine or noradrenaline carboxylic acid.

(3) *Experiments with* Streptococcus faecalis

It is known that the *L*-tyrosine decarboxylase of *Strep. faecalis* also acts on 3:4-dihydroxyphenylalanine (Epps, 1944). It seemed therefore of interest to find out how the introduction of a hydroxyl group in the β-position of the side chain would affect substrate specificity.

Two experiments were carried out in which the amino-acid was incubated with the bacterial preparation. Carbon dioxide was formed in both experiments. This suggested that noradrenaline had been formed by the enzyme. The pressor activity of the solutions was therefore estimated on the blood pressure of the spinal cat. In the first experiment the activity was assayed against (±)-noradrenaline and in the second against the laevorotatory isomer. Since the results obtained in the two experiments showed satisfactory agreement, only the second experiment will be described in detail.

(*a*) *Incubation.*—A suspension was prepared containing 10 mg. of the acetone-dried bacteria in 1 ml. of distilled water.

Two manometer flasks were set up as follows:

	Blank	*Test*
Main Flask	1.0 ml. bacterial suspension + 0.1 ml. M/1 acetate buffer, *p*H 5.5 + 0.2 ml. water in both flasks	
Side Bulb	0.2 ml. water	0.2 ml. M/12.5 noradrenaline carboxylic acid

A third flask was set up in which the noradrenaline carboxylic acid in the side bulb was replaced by 0.2 ml. of a suspension of *L*-tyrosine. This flask was used in order to enable us to relate the rate of decarboxylation of noradrenaline carboxylic acid to that of tyrosine.

The incubation was carried out at a temperature of 28.5° C.

Separately, a smaller amount of *L*-tyrosine was incubated with the bacterial preparation under the same conditions; this was done in order to determine how much of the carbon dioxide formed had been retained as bicarbonate. The retention was found to amount to about 6 per cent; this figure was therefore used as a correction in the main experiment.

Formation of carbon dioxide occurred when noradrenaline carboxylic acid was added to the enzyme. The rate of decarboxylation was steady for the first hour, but then slowed down; the reaction had almost come to a standstill when the incubation was stopped after 315 min. By that time 168 μl. of carbon dioxide had been formed. On the assumption that half a molecule of carbon dioxide is formed from one molecule of noradrenaline carboxylic acid 179 μl. should be formed. Thus the observed formation of carbon dioxide represents 94 per cent of theory. The time course of the reaction is shown in Fig. 1.

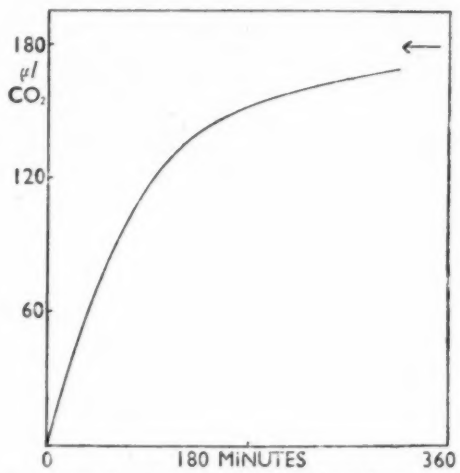


FIG. 1.—Decarboxylation of noradrenaline carboxylic acid by *Streptococcus faecalis* R. Abscissa: time in minutes; Ordinate: μl. CO_2 formed. The arrow denotes half a molecule of CO_2 per molecule of amino-acid added.

After the last reading the contents of the blank and test flasks were each pipetted into a centrifuge tube. Each flask was washed out with two successive portions of 0.25 ml. of water. For the pharmacological assay it was assumed that any active material formed was now contained in a total volume of 2.0 ml.

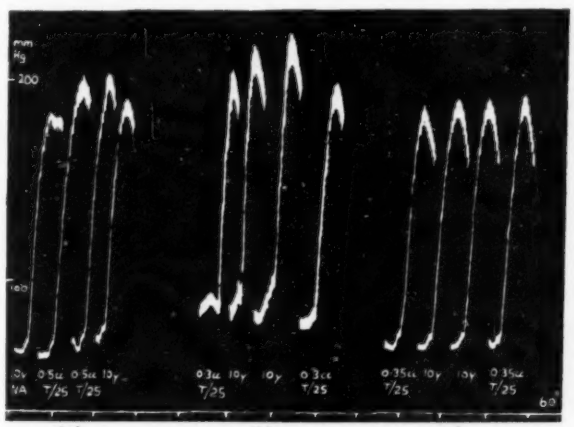
The suspensions were centrifuged for 15 min., in order to remove the bacterial debris, and the supernatant fluids were used for the assay.

(*b*) *Assay.*—The manometric experiment suggested that amine formation from noradrenaline

carboxylic acid had occurred. It was desirable to establish the identity of the amine formed with noradrenaline and to determine its pharmacological activity. The assay was carried out on the spinal cat and the identity of the pressor principle was confirmed on the rat's uterus.

Spinal cat; 3.2 kg.; ♂. The test was used in a dilution 1 in 25. The standard of laevorotatory noradrenaline was made up in a solution of the blank so that the final concentration of the blank was also 1 in 25.

The pressor activity of the test sample is shown in Fig. 2; the tracing shows that 0.5 ml. of the test,



(*a*) (*b*) (*c*)

FIG. 2.—Assay of pressor activity produced from noradrenaline carboxylic acid by the bacterial enzyme. Arterial blood pressure of the spinal cat. 10 μg. of synthetic (−)-noradrenaline were: (*a*) less active than 0.5 ml., (*b*) more active than 0.3 ml., (*c*) about as active as 0.35 ml. of the test solution, diluted 1 in 25.

diluted 1 in 25, had a stronger, and 0.3 ml. of the same solution a weaker, pressor action than 10 μg. of (−)-noradrenaline. The pressor action of 10 μg. of (−)-noradrenaline was found to be approximately equal to that of 0.35 ml. of the test, diluted 1 in 25. The blank was without action on the blood pressure.

(*c*) *Calculation of result.*—The total amount of pressor activity formed in the experiment can be calculated from these data. The pressor action contained in 2 ml. of the undiluted test was equivalent to more than 1.00 mg. and less than 1.62 mg. of (−)-noradrenaline, and about equal to 1.40 mg. of (−)-noradrenaline.

The total amount of amine expected to be present can be calculated from the amount of carbon dioxide formed; one molecule of amine is formed per molecule of carbon dioxide:

$$R\text{-}CH(NH_2)COOH \longrightarrow R\text{-}CH_2NH_2 + CO_2$$

The molecular weight of noradrenaline is 169. Therefore, 22,400 μl. of CO_2 correspond to 169 mg. of noradrenaline, and 168 μl. of CO_2, the amount formed in the experiment, correspond to

$$\frac{169 \times 168}{22{,}400} = 1.27 \text{ mg. of noradrenaline.}$$

The agreement between the amounts found and expected is satisfactory and shows that the amine formed was in fact (−)-noradrenaline. The amine formed cannot have been (±)-noradrenaline.

Similarly, it could be calculated that in the first experiment, in which the activity was assayed against (±)-noradrenaline, the amount of amine formed was about twice as active as (±)-noradrenaline. In this experiment the assay on the cat's blood pressure was followed by an assay on the rat's uterus.

(*d*) *Rat's uterus assay.*—One horn of the uterus of a non-pregnant rat was suspended in a bath of capacity 5 ml. at a temperature of 31° C. The composition of the Ringer's fluid was similar to that recommended by Garcia de Jalon *et al.* (1945). Submaximal contractions of the uterus were recorded; a dose of 5 μg. of acetylcholine was added to the bath every 90 secs. The acetylcholine was present for 30 secs., and 60 secs. were allowed for recovery. Adrenaline, noradrenaline, or the test solution was added to the bath 30 secs. before the acetylcholine.

(*a*) (*b*) (*c*)

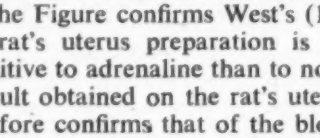
FIG. 3.—Isolated uterus of the rat. Each contraction was caused by 5 μg. of acetylcholine. The inhibitions were due to: (*a*) the test solution in an amount calculated to contain 0.4 μg. of amine, (*b*) 0.8 μg. of (±)-noradrenaline, (*c*) 0.004 μg. of (−)-adrenaline.

A dilution of the test was given calculated to contain 0.4 μg. of (−)-noradrenaline. Fig. 3 shows that the inhibition observed was about equal to that after 0.8 μg. of (±)-noradrenaline. The same result was obtained at three different dose levels. The Figure confirms West's (1947) finding that the rat's uterus preparation is many times more sensitive to adrenaline than to noradrenaline.

The result obtained on the rat's uterus preparation therefore confirms that of the blood pressure assay: the amine formed is (−)-noradrenaline.

Discussion

In order to assess whether or not noradrenaline carboxylic acid can be considered as a likely precursor of adrenaline or sympathin, it is necessary to consider its structure. The acid differs from 3:4-dihydroxyphenylalanine in that it contains 2 asymmetric carbon atoms, one in position α and one in position β.



Noradrenaline carboxylic acid

Compounds of this kind have four different configurations and exist in pairs related as object and mirror image. The four possible arrangements in space are shown in Fig. 4, in which the α and β carbon atoms are replaced by regular tetrahedra. The designations *L* and *D* are used for the α carbon atom: the configuration *L* is that common to most naturally occurring amino-acids. The two possible configurations of the β carbon atom are distinguished as *l* and *d*; this is the asymmetric carbon atom of adrenaline; the naturally occurring laevorotatory configuration is usually referred to as *l*-adrenaline.

Dr. Mann told us that the sample of noradrenaline carboxylic acid which we used in our experiments behaved like a simple racemic mixture and that it therefore contained only one pair of stereoisomers. The two pairs of stereoisomers are *Dd–Ll* and *Dl–Ld*.

The experiments reported above enable us to determine the configuration of noradrenaline carboxylic acid. This is possible, because two stereospecific affinities are involved:

(*a*) that of the bacterial amino-acid decarboxylases for the *L*-configuration of the α carbon atom, and

(*b*) that of the plain muscle cell for the *l*-configuration of the β carbon atom.

All known amino-acid decarboxylases, bacterial and mammalian, are stereospecific, and it can therefore be safely assumed that only the *L*-stereoisomer was decarboxylated in our experiments. This is borne out by the observation that approximately half a molecule of carbon dioxide was formed from one molecule of noradrenaline carboxylic acid.

The stereospecificity of the mammalian receptors has recently been studied by Tainter, Tullar, and Luduena (1948), who have shown that on the spinal cat's blood pressure the ratio of equiactive doses of laevorotatory to dextrorotatory noradrenaline is 1:25 to 1:33.

Theoretically three structures are possible:

(1) a racemic mixture of the configurations *Dd* and *Ll*; in this case one would expect (−)-noradrenaline to be formed.

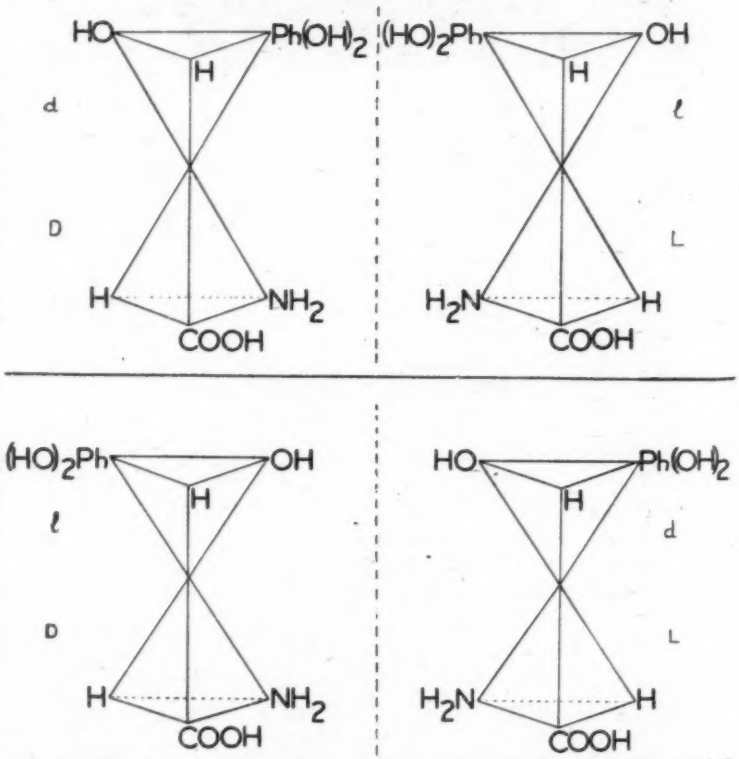


FIG. 4.—The four configurations of "noradrenaline carboxylic acid." The alpha carbon atoms are represented by the lower, and the beta carbon atoms by the upper, tetrahedra.

(2) a racemic mixture of the configurations *Dl* and *Ld*; in this case one would expect (±)-noradrenaline to be formed.

(3) a mixture of all four configurations: in this case one would expect (±)-noradrenaline to be formed. (This possibility was already ruled out by Dr. Mann's findings.)

That the first of these is in fact the structure of the acid is proved by the pharmacological assay on the spinal cat. The *L*-amino acid was found to be almost quantitatively decarboxylated and to have given rise to (−)-noradrenaline.

Rules for the nomenclature of amino-acids have recently been agreed upon by the editors of the *Biochemical Journal*, the *Journal of the Chemical Society*, and the corresponding American publications (1948). Rule 6 deals with amino-acids "with two asymmetric centres, but where internal compensation is impossible." Noradrenaline carboxylic acid is unlikely to be present in proteins, but it is so closely related to amino-acids found in proteins that it seems desirable to follow the rules laid down for these compounds. The substance studied in this paper contains the configuration in which the *L*-amino-acid has the configuration related to naturally occurring laevorotatory adrenaline, and it is therefore proposed to use the designation 3:4-dihydroxyphenylserine for this specimen and to reserve the prefix allo- for the pair which still awaits synthesis.

3:4-Dihydroxyphenylserine has the configuration to be expected in a possible precursor of adrenaline, so that the negative results obtained with the mammalian decarboxylases cannot be ascribed to the use of the wrong stereoisomer but must be interpreted as meaning that this substance is unlikely to be a precursor of adrenaline or sympathin.

Summary

(1) Noradrenaline carboxylic acid is not a substrate of dopa decarboxylase.

(2) Extracts of guinea-pig's suprarenal glands do not contain dopa decarboxylase, and they do not decarboxylate noradrenaline carboxylic acid.

(3) Noradrenaline carboxylic acid was decarboxylated by an acetone-dried preparation of *Streptococcus faecalis* R; the amine formed was characterized as (−)-noradrenaline by pharmacological assay on the blood pressure of the spinal cat and on the rat's uterus.

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ACTIVATION OF PALUDRINE

BY

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The purpose of this paper is to submit evidence that paludrine has little activity in preventing the development of malaria parasites *in vitro*, but that it develops such activity when administered to the mammalian or avian host or when incubated with minced liver tissue.

Paludrine is N_1-*p*-chlorophenyl-N_5-*iso*propyl biguanide.

Tonkin (1946), in this laboratory, investigated the effect of adding paludrine to tissue cultures containing exo-erythrocytic forms of *Plasmodium gallinaceum* and found that the parasites developed in the highest concentrations (2–5 mg. per litre) tolerated by the macrophages. Since this concentration is many times greater than that which is believed to be present in the plasma during human therapy (probably about 0.3 mg. per litre), and since Davey (1946) had reported that paludrine was highly effective in curing the exo-erythrocytic infections of *P. gallinaceum* in chickens, as well as the endo-erythrocytic ones, Tonkin's finding was surprising and led to further investigations, which were undertaken with both *P. gallinaceum* and *P. cynomolgi*. A preliminary note describing some of the results was published by Hawking (1947).

Experiments with P. gallinaceum *in tissue culture*

The exo-erythrocytic forms of *P. gallinaceum* were grown in tissue culture at 37° C. by the technique described by Hawking (1945) and used by Tonkin (1946) to study the action of anti-malarial compounds. The implants were obtained from the spleen of chickens, at the stage of infection when exo-erythrocytic parasites were present. In the first experiments the cultures were grown in the presence of normal serum for several days, until the development of parasites in them could be demonstrated; the medium to be tested for antimalarial activity was then added and the fate of the parasites was observed by removing cultures at intervals during the next ten days for staining with Giemsa and examination in the usual way. The protocol of a typical experiment is reproduced in Table I. For this experiment serum was

TABLE I

The medium consisted of 20 per cent serum, 20 per cent embryo extract, and 60 per cent Tyrode's solution by volume. The paludrine serum in Nos. 2, 3, and 4 was substituted for all or part of the normal serum. The paludrine in Nos. 6 and 7 was added to the Tyrode's solution. On the 5th day the medium was changed, and fresh medium similar to that originally present was added. Each No. represents two Carrel flasks and most of the observations are based on four cell-colonies (slips), two from each flask. The cultures had grown for 4 days before the experiment began, and the development of parasites in them had been demonstrated.

No.	Contents	Presence of parasites in cultures		
		2nd day	4th day	7th day
1	Control—Normal serum 20%	—	—	Many
2	Paludrine serum 20%	—	A few in one culture. None in another	None—chick not infected
3	Paludrine serum 7%	Some	None	None—chick not infected
4	Paludrine serum 2%	Some	None	None
5	Paludrine 5 mg. per l.	—	—	Toxic to cells
6	Paludrine 2 mg. per l.	—	—	Many
7	Paludrine 1 mg. per l.	—	—	Many

obtained from a fowl to make up the normal fluid culture medium. The fowl was then given 300 mg. paludrine per kg. intramuscularly (a toxic dose), and blood was removed 2¼ hours later to provide the "paludrine serum." (All quantities of paludrine in this work refer to paludrine acetate.)

In this experiment paludrine added directly to tissue cultures had little or no action on the parasites unless the final concentration was so high (e.g., 5 mg. per litre in No. 5) as to be toxic to the cells without which the parasites cannot grow; this reproduces the finding of Tonkin already mentioned. On the other hand the addition of serum from a fowl treated with a large dose of paludrine was highly active, and all the cultures were sterilized, mostly by the 4th day. The survival of parasites for a few days, even in a medium which eventually kills them, is in agreement with the experience of Tonkin, who found that parasites persisted in the presence of sulphathiazole (5–50 mg. per litre) for 4 days but that most were destroyed by the 5th day. The death of the parasites in Nos. 2 and 3 was confirmed by removing fluid from the flasks on the 7th day and injecting it into chicks, which did not become infected. Probably the activity shown by the paludrine-serum in this experiment was particularly high because of the large size of the dose of paludrine given to the fowl. In other experiments the fowl was given 30 mg. per kg. 18 hours and 2 hours before the blood was taken, and the antimalarial action of the serum was not manifested when the concentration of serum in the flask was less than 5 per cent.

These results suggest that paludrine undergoes some modification in the fowl so that it develops an activity against the parasites *in vitro* which was not possessed by the original paludrine. This hypothesis was studied further by incubating paludrine with minced liver tissue and then testing its action on the parasites. The liver was removed aseptically from normal rats and minced finely. About 6 c.c. of minced liver was placed in each of two tubes containing 10 c.c. of Ringer with 0.2 per cent (w/v) glucose; one tube contained 12 mg. paludrine per litre, which when diluted 1 in 6 provided a final concentration of 2 mg. per litre, and the other served as a control. The tubes were incubated at 37° C. for 4 hours and then centrifuged, and the supernatants removed. These provided the "liver extract" and "liver extract plus paludrine," the use of which is illustrated in the typical protocol reproduced in Table II. In this experiment the fluids to be tested for antimalarial activity were added to the Carrel flasks when the cultures were first set up, without allowing a preliminary period for growth (Tonkin, 1946). Serum A was obtained from a normal fowl and it was used as serum for all the cultures unless stated otherwise. Serum B_1 was obtained from a fowl *before* it received paludrine; it was then given two intramuscular injections of 30 mg. per kg. 18 hours and 1 hour respectively before the "paludrine serum," B_2, was withdrawn. In this experiment the paludrine serum showed fairly high activity, since the growth of parasites was prevented (completely or incompletely) by concentrations of 1–4 per cent. Liver extract alone had no activity against the parasites; but paludrine which had been incubated with liver showed fair activity, preventing the growth of all but rare parasites when in a concentration originally of 2 mg. per litre. Actually, it is probable that much of the paludrine had been absorbed by the liver tissue and the real concentration of paludrine (and paludrine derivatives) was much less than this theoretical figure. Other experiments (see Table I) have demonstrated that unchanged

TABLE II

The medium consisted of 17 per cent serum, 4 per cent embryo extract, and 79 per cent Tyrode's solution by volume. The paludrine serum in Nos. 3, 4, and 5 was substituted for all or part of the normal serum (A). The liver extract (with or without paludrine) in Nos. 6, 7, 8, and 9 was substituted for part of the Tyrode's solution. Each No. represents two Carrel flasks and most of the observations are based on four cell-colonies (slips).

No.	Contents	Concentration % (v/v)	Presence of parasites on 5th day
1	Serum A Control	17	Many
2	Serum B_1 Control	17	Very many
3	Paludrine serum B_1	17	None
4	Paludrine serum B_2	4	Few and degenerate
5	Paludrine serum B_2	1	None
6	Liver extract	17	Very many
7	Liver extract + paludrine	17 + 2 mg. per l.	None in 2 cultures, 1 parasite in a 3rd culture, and 2 in a 4th
8	Liver extract + paludrine	4 + 0.5 mg. per l.	Fair number
9	Liver extract + paludrine	1 + 0.12 mg. per l.	Few

paludrine is inactive in a concentration of 2 mg. per litre.

After the tissue culture experiments had been completed, chemical estimations were made on the concentration of paludrine present in the serum of two fowls, each of which had received two intramuscular injections of 30 mg. paludrine per kg. 18 hours and 1 hour respectively before the blood was withdrawn. The concentrations found were 11 mg. and 27 mg. per litre respectively, average 19±11.3 mg. per litre. As the serum constituted only 17 per cent of the fluid present in the flasks of Table II, the highest concentration of paludrine present in the medium containing "paludrine-serum" (Flask 3) would be about 3.2 mg. per litre; while the medium in Flask 5 (which contained only 1 per cent paludrine serum and which prevented growth of the parasites) would contain 0.19 mg. per litre. These figures may be compared with the concentration of 2 mg. unchanged paludrine per litre which is inactive.

Experiments with P. cynomolgi

P. cynomolgi was cultured *in vitro* at 37° C. by a modification of Bass and John's technique. Briefly, about 15 c.c. blood was withdrawn from a rhesus monkey (A) infected with *P. cynomolgi* at a time, preferably before noon, when most of the parasites (95 per cent) were present as small rings. The blood was defibrinated by shaking with glass beads. It was centrifuged, most of the serum was removed, the corpuscles being resuspended in the remainder. Sufficient 10 per cent (w/v) glucose solution was added to the serum to make the concentration of added glucose 0.2 per cent. The cultures were made in small flat-bottomed tubes, 6 cm. high by 0.6 cm. internal diameter, which were closed by rubber bungs. Each tube contained 0.6 c.c. monkey serum, 0.1 c.c. Ringer's solution, and 0.05 c.c. suspension of parasitized red blood corpuscles. The corpuscles formed a thin layer on the floor of the tube. Serum A was obtained from Monkey A or from another normal

TABLE III

Serum A was obtained from an untreated monkey which provided the parasitized blood. Serum B_1 was taken from a second monkey before it received paludrine. Serum B_2 was taken from the second monkey at 11 a.m., when it had received paludrine 50 mg. 18 and 2 hours previously. In tubes 9–11 the dilutions were made with serum A. Tubes 12–19 contained serum A (0.6 c.c.) plus 0.1 c.c. of liver extract (incubated with or without paludrine 14 mg. per litre) pure or in various dilutions made with Ringer. At the beginning of the experiment all the parasites contained only one piece of chromatin, and 95 of them were young rings; there were no gametocytes.

Tube	Contents	Per cent distribution of parasites according to number of pieces of chromatin					
		1	2–3	4–5	6–8	9–16	Degenerate
1	Control, serum A	26	22	12	16	24	0
2	,, ,, ,,	32	18	14	10	26	0
3	Control, serum B_1	28	22	18	10	22	0
4	,, ,, ,,	36	16	4	16	28	0
5	Paludrine, added to serum A, 2 mg. per l.	26	23	15	11	25	0
6	Paludrine, added to serum A, 1 mg. per l.	35	25	10	10	20	0
7	Serum B_2 from monkey treated with paludrine, pure	42	18	6	4	0	30
8	—ditto—pure	54	28	4	0	0	14
9	—ditto—diluted 1 in 4	48	40	8	0	0	4
10	—ditto—diluted 1 in 16	28	48	4	0	0	20
11	—ditto—diluted 1 in 64	69	22	3	0	0	6
12	Liver extract added, pure	23	42	15	7	13	0
13	—ditto—diluted 1 in 4	15	25	9	6	12	33
14	—ditto—diluted 1 in 16	35	20	15	10	20	0
15	Paludrine + liver extract, pure (2 mg. per l.)	4	6	0	0	2	88
16	—ditto—pure (2 mg. per l.)	10	0	0	0	0	90
17	—ditto—diluted 1 in 4 (0.5 mg. per l.)	52	22	6	4	2	14
18	—ditto—diluted 1 in 16 (0.125 mg. per l.)	48	24	4	0	0	24
19	—ditto—diluted 1 in 64 (0.03 mg. per l.)	36	24	16	8	16	0

monkey. A sample of serum (B_1) was collected from another monkey (B). This served as a control to exclude non-specific antimalarial activity. Monkey B was then treated with paludrine, receiving usually two intramuscular injections of 50 mg. for a 3 kg. animal at 5 p.m. and 9.30 a.m.; two hours after the last injection blood was collected from a vein and defibrinated; this provided serum B_2 for the cultures. In the experiments with liver extract paludrine was incubated with minced liver exactly as described above. The concentration of paludrine used, however, was 14 mg. per litre, so that when 0.1 c.c. was added to 0.6 c.c. medium in the culture tube the final theoretical concentration would be 2 mg. per litre. In the tubes to which paludrine was added the paludrine was dissolved in Ringer's solution and added in place of the normal Ringer's solution (0.1 c.c.) in the control tubes. Precautions to maintain sterility were observed throughout. The tubes were incubated at 37° C. After 20 and 42 hours a small sample of the corpuscles was removed by a pipette and smeared out to form thin blood films which were fixed in alcohol and stained with Giemsa. The parasites were examined and classified according to the number of pieces of chromatin which they contained, the identity of the slide being unknown to the investigator during examination.

The protocol of a typical experiment is reproduced in Table III, which shows:

(1) That many of the parasites develop normally (as shown by the division of chromatin) in the presence of serum from the monkey which supplied the parasitized corpuscles (tubes 1 and 2), serum from a second monkey (tubes 3 and 4), or liver extract (tubes 12–14).

(2) That the development of the parasites is not prevented by the addition of paludrine to the cultures sufficient to produce a concentration of 1 or 2 mg. per litre (tubes 5 and 6); in other experiments the parasites developed normally in a concentration of 20 mg. paludrine per litre.

(3) That the development of the parasites is almost completely prevented by serum from a monkey which has received paludrine during the previous 20 hours; this serum is active even when diluted 64 times (tubes 7–11). The parasites in the tubes containing activated paludrine showed various degeneration changes which are described below.

(4) That the development of parasites is similarly prevented by the addition of paludrine which has been incubated with liver (tubes 15 and 18), although the addition of liver extract alone does not inhibit development (tubes 12–14). Inhibition occurs when the paludrine + liver extract is diluted 16 times (tube 18; paludrine theoretically 0.12 mg. per litre) but not when diluted 64 times (tube 19; paludrine theoretically 0.03 mg. per litre).

After these experiments had been completed chemical estimations were made of the concentration of paludrine present in the serum of three monkeys which had been previously treated with paludrine, each receiving 50 mg. per monkey 18 hours and 2 hours before the serum was obtained. The results obtained, using a modification of the method of Spinks and Tottey (1946), are shown in Table IV.

TABLE IV

Monkey	Weight (kg.)	Dose (mg. per kg.)	Blood paludrine (mg. per litre)
1	5.8	8.6	20
2	2.5	20 0	34
3	3.6	13.9	12

The average concentration of paludrine in the serum of a monkey receiving 50 mg. of paludrine as above was thus 22 ± 10.7 mg. per litre. This level is approximately equal to the highest concentration of paludrine (20 mg. per litre) tested in the control tubes during this work; as recorded above, 20 mg. per litre of paludrine solution did not affect the normal development of the parasites. The medium in Tube 11 of Table III was fully active in preventing the development of the parasites although it contained only 1 part of paludrine serum in 64—i.e., probably about 0.3 mg. paludrine per litre.

The conclusions derived from this work are similar to those derived from the experiments with the tissue cultures of *P. gallinaceum*, viz. that paludrine itself (even in a concentration of 20 mg. per litre) is not active in preventing the development of the parasites *in vitro* but that it becomes active when exposed to the cells of the body (as in the treated monkey) or to minced liver tissue.

In a further experiment the supernatant fluid from the incubated tube of paludrine plus liver tissue was heated to 100° C. for 20 minutes in order to precipitate all the protein, which was removed by filtration. This filtrate was as active as the unheated supernatant, which indicates that the active derivative of paludrine is probably not bound to protein, and is probably not thermolabile.

Attempts were made to develop a simple technique for testing the activity of paludrine derivatives *in vitro* by incubating blood containing endo-erythrocytic forms of *P. gallinaceum* with paludrine serum or paludrine + liver extract for several hours and then injecting it into chickens to test for infectivity. The results were very irregular, and most of the specimens were able to infect chickens in spite of exposure to paludrine or its hypothetical products. Accordingly these attempts were abandoned.

Morphological description of degenerate parasites

Examination of preparations of *P. cynomolgi* from cultures in which the parasites had been subjected to the action of activated paludrine showed that, in addition to a failure of development to mature schizonts, the parasites suffered a series of degenerative changes.

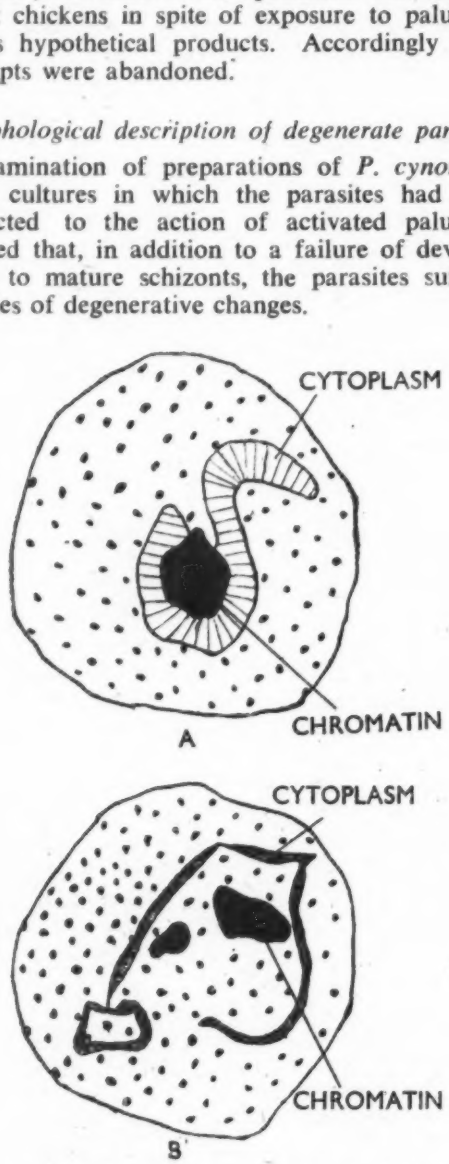

FIG. 1.—Malaria parasites (*P. cynomolgi*) showing degeneration produced by paludrine serum. A. The chromatin is small and dark and the cytoplasm is thinned out and translucent. B. The chromatin is condensed into two dark masses; the cytoplasm is disrupted into narrow strands scattered throughout the corpuscle. Magnification about 8,000.

A few of the parasites were small, containing one piece of very darkly staining chromatin and having a minimum of cytoplasm, also darkly stained, closely packed round the chromatin. Others showed the same small mass of dense chromatin, but had a varying amount of poorly stained pinkish cytoplasm thinned out and almost translucent, so that it was difficult to differentiate from the surrounding corpuscle. An example of this type of parasite is illustrated in Fig. 1A. In this particular specimen the cytoplasm was thin and translucent, but not so distended as in others, in which, indeed, the cytoplasm occupied almost the whole corpuscle. Many parasites, however, exhibited a more characteristic series of degenerative changes than those just described. A typical example is shown in Fig. 1B. The chromatin was condensed into one or more very darkly staining lumps. The cytoplasm was disrupted, and was often arranged in narrow dark-staining strands, which were scattered throughout the corpuscles and woven sometimes into fantastic patterns; frequently the strands were completely separated from the chromatin masses, which were left denuded of any cytoplasmic covering. This series of changes was more common in samples withdrawn from culture tubes after incubation for 42 hours than in samples withdrawn after only 20 hours. The parasitized red blood corpuscles showed the stippling usual with *P. cynomolgi* infections. This series of degenerative changes observed *in vitro* may be compared with the changes undergone by *P. vivax* when subjected to the action of paludrine *in vivo* (Mackerras and Ercole, 1947).

DISCUSSION

The experiments described have shown that paludrine in a concentration of 20 mg. per litre has no apparent action *in vitro* on endo-erythrocytic forms of *P. cynomolgi*; similarly a concentration of 2 mg. per litre has no action on the exo-erythrocytic forms of *P. gallinaceum*. If, however, paludrine has been previously exposed to the action of body cells, either by injecting it into a monkey or fowl and collecting the serum, or by incubating it with minced rat liver, marked antimalarial action can be demonstrated. The simplest explanation for these facts is that paludrine undergoes some chemical modification which converts it into an active compound. This hypothesis would explain the contradictory findings of Tonkin (1946) that paludrine had no antimalarial action when added to tissue cultures of exo-erythrocytic forms of *P. gallinaceum*, and of Black (1946), who reported that serum from a patient treated with

paludrine arrested the development *in vitro* of trophozoites of *P. falciparum*. Marshall (1947) has reported that paludrine solutions are active in inhibiting the uptake of oxygen by *P. gallinaceum in vitro*; but the concentrations of paludrine required to produce this effect were 10^4 to 10^5 times as great as those used in our experiments (1 in 3,000 to 1 in 6,000 as compared with 1 in 1,000,000 to 1 in 10,000,000). No work has yet been reported on the corresponding activity of paludrine after activation by the living animal or by liver extract.

A similar conversion to an active substance occurs with tryparsamide and other pentavalent arsenicals which are converted by cells from the inactive pentavalent to the active trivalent state. With paludrine it is not easy to suggest the nature of the hypothetical chemical modification. According to Acheson, King, and Spensley (1947) it is unlikely that activation depends on the conversion of paludrine into a benziminazole. Our present information indicates that this activation can be carried out by the whole organism or by minced liver; it cannot be produced by the red blood corpuscles or by the macrophages and fibroblasts present in tissue cultures from the spleen. The further study of the activation of paludrine requires a simpler and more convenient technique for measuring the antimalarial action of compounds *in vitro*. The methods used in the present work, although adequate to demonstrate the occurrence of activation, are rather laborious and insensitive for use in a detailed investigation of the phenomenon.

The conversion of paludrine by cells into an active derivative is of importance in the study of the action of paludrine *in vitro*, the relation of chemical structure to activity, the search for more active antimalarial compounds, the interpretation of chemical estimations of the blood concentrations of paludrine, and other similar questions; most of this is obvious and does not require elaboration.

Summary

1. No demonstrable antimalarial action is exerted *in vitro* on cultures of the exo-erythrocytic forms of *Plasmodium gallinaceum* by paludrine in concentrations of 2 mg. per litre, or on endo-erythrocytic forms of *P. cynomolgi* by paludrine in concentrations up to 20 mg. per litre; these concentrations are higher than those which are commonly reached in the blood during human therapy.

2. Serum from a fowl or monkey, which has recently received paludrine, exercises a pronounced action on these cultures, preventing the development of the parasites; cultures of *P. gallinaceum* are destroyed.

3. Paludrine which has been incubated with minced liver has a similar pronounced antiplasmodial action *in vitro*.

4. These phenomena suggest that paludrine itself is not active against plasmodia but that it can somehow be modified by the body or by liver cells, so that it becomes actively plasmodicidal.

Our thanks are due to Mr. E. C. England and Miss J. Barten for technical assistance.

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A COMPARISON OF THE IRRITANT ACTION OF CONESSINE, *ISO*CONESSINE, AND *NEO*CONESSINE

BY

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Conessine is a powerful local anaesthetic, but its use is limited since its injection produces inflammation which may be followed by necrosis. *Iso*conessine and *neo*conessine are isomers of conessine and they also possess local anaesthetic actions. It was therefore of interest to compare their irritant action with that of conessine.

No method for comparing the irritant action of different substances in animals has hitherto been described and a method has been used which is in some respects similar to the guinea-pig skin test for diphtheria antitoxin and for old tuberculin. In a preliminary experiment 0.2 ml. of 1 in 50, 1 in 100, 1 in 200, 1 in 400, and 1 in 800 solutions of conessine dihydrochloride were injected intracutaneously into a guinea-pig and the weals produced by the injection were ringed with ink. During the next three days no effect was apparent at the site of injection of the 1 in 800 solution, while the 1 in 400 solution produced only a very slight inflammation. The higher concentrations produced progressively greater effects and at 72 hours the site of the injection of the 1 in 50 solution was covered by scar tissue. The fact that these concentrations produced an appreciable and graded response indicated that a comparison could be made in this way.

In order to compare the effects produced by the different substances, guinea-pigs were prepared by removing the hair from the back with electric clippers. The back was marked out into 6 areas, 3 on each side of the spine, and 0.2 ml. of each of 3 concentrations of one alkaloid (1 in 50, 1 in 100, and 1 in 200) were injected on one side and the same concentrations of a different alkaloid on the other side. In half the animals the solutions were injected in increasing strength towards the head and in the other half this order was reversed. Each concentration of each alkaloid was injected into 6 animals. Inflammation appeared within about 2 hours and a first attempt to compare the effects was made between 2–4½ hours after the injection. Scores from 0 to 3 were awarded according to the amount of inflammation. After 24 hours the inflammation was more marked and scar tissue was beginning to appear. Estimates of the effects were again made, the range of scores being increased in accordance with the increased effect. After 72 hours the inflammation had largely disappeared and was replaced by scar. Scores from 0 to 6 were again given to the different areas. Two other observers also examined the areas and allotted scores to each. Their results were similar to those already obtained.

The mean result for each concentration of each alkaloid was plotted as an ordinate against the concentration as abscissa (using a logarithmic scale) in Fig. 1, in which the relation between the alkaloids

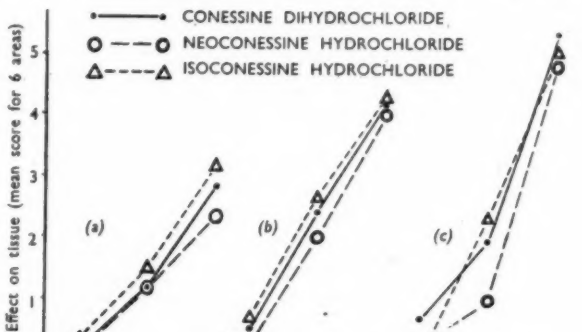

FIG. 1.—Graphs showing the estimates made by one person of the amount of inflammation and necrosis after the injection of varying concentrations of conessine and its isomers. (*a*) 2–4½ hours after injection; (*b*) 24 hours; (*c*) 72 hours.

is shown as estimated at (*a*) 2–4½ hours, (*b*) 24 hours, and (*c*) 72 hours after injection. Fig. 1 shows that *neo*conessine causes slightly less inflammatory reaction than the others, but the difference is very small. All three substances begin to pro-

duce inflammation in a concentration of 1 in 200. The smallest concentration producing local anaesthetic action is in the range 1 in 6,400 to 1 in 3,200 (Stephenson, 1948); this, however, is a very slight degree of anaesthesia. More is obtained in the range 1 in 1,600 to 1 in 800, which is still well below the concentration causing inflammation.

Solutions containing adrenaline.—Procaine is commonly mixed with adrenaline, and its local anaesthetic action is greatly increased thereby. It seemed possible that the local anaesthetic action of conessine might also be increased if adrenaline were mixed with it, and a comparison was therefore carried out in which solutions of conessine were compared with similar solutions containing adrenaline. Six guinea-pigs were used, and the results obtained by the method of Bülbring and Wajda (1945) are shown in Table I.

TABLE I

0.2 ml. solution is injected intradermally. The area is pricked 6 times at 5, 10, 15, 20, 25, and 30 min. after injection. The number of times there is failure to respond is observed and is shown below. The maximum number of failures is 36

Pig No.	Conessine			Conessine + Adr. 1 in 100,000		
	1 in 830	1 in 1660	1 in 3320	1 in 830	1 in 1660	1 in 3320
1	36	25	15	35	34	30
2	21	20	10	29	27	20
3	29	26	27	33	20	12
4	35	22	15	35	28	15
5	35	26	26	36	28	21
6	29	20	0	35	32	25
Mean	31	23.1	15.5	33.8	28.1	20.4

Table I shows that the presence of adrenaline caused some increase in the duration of anaesthesia produced by conessine, but less than that with procaine. The difference can be expressed by saying that the addition of adrenaline would enable a solution containing 1 in 1,000 conessine dihydrochloride to act like a solution containing 1 in 700.

In view of the fact that the work of R. D. Haworth and Nazar Singh (1948) indicates that conessine is a steroid, it may well be that it is less readily absorbed from the site of injection than procaine. Thus the fact that adrenaline does not so greatly intensify its local anaesthetic action within the period of 30 min. observation is not surprising.

Experiments were also made to observe whether the addition of adrenaline to a solution of conessine would alter the irritant action of the solution. Solutions of conessine dihydrochloride containing adrenaline 1 in 100,000 were compared with the same solution, without adrenaline. The conessine solutions were 1 in 400, 1 in 200, and 1 in 100; each solution was injected on one side of the back of a guinea-pig and compared with the same solution containing adrenaline on the other side. Two animals were used, and in neither was any difference observed owing to the presence of adrenaline. The 1 in 100 solution produced obvious inflammation, followed by necrosis. The 1 in 200 solution produced less inflammation and an induration at the site of injection.

SUMMARY

A method of making a quantitative comparison of two or more substances for local irritant action is described. Conessine, *iso*conessine and *neo*conessine all begin to cause inflammation after intracutaneous injection in a concentration of 1 in 200. The local anaesthetic action of conessine is intensified when adrenaline (1 in 100,000) is present by about 40 per cent. The presence of adrenaline does not affect the irritant action of conessine.

This work was done when one of us (R. P. S.) was in receipt of a grant from the Therapeutic Research Corporation.

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A MODIFICATION OF THE METHOD OF DALE AND LAIDLAW FOR STANDARDIZATION OF POSTERIOR PITUITARY EXTRACT

BY

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The activity of a posterior pituitary extract is still generally determined by the method introduced by Dale and Laidlaw (1912). They used the uterus of a virgin guinea-pig, and the assay consisted in setting limits to the potency of the test solution in terms of the standard solution by the usual ABBA grouping of doses. The method suffered from three defects.

1. Suitable guinea-pigs were relatively scarce.

2. The assay often required many hours.

3. The error of the method was about 20 per cent (Gaddum, 1938).

A different method of assay was described by Thompson (1944). He used the relationship between the dose of pituitary and the fall in blood pressure in the chicken which had been investigated by Smith and Vos (1943). Thompson obtained results with very small errors and was able to assess the reliability of each result from the experimental data. He claimed that the method was rapid and easy to perform. There is, however, one drawback to the method. It is possible that the active principle responsible for the fall in blood pressure is not identical with that which makes the uterus contract. Thompson himself found a significant difference between potency on the blood pressure and potency on the guinea-pig uterus in 1 out of 17 parallel determinations and suggested that the blood pressure method cannot always be relied upon to estimate the oxytocic activity.

It seemed best, therefore, to return to the original method of standardization using the isolated uterus as a test preparation. Various modifications of the original method have recently been introduced. Morrell, Allmark, and Bachinski (1940) used 8 strips of muscle which were cut from the same uterus and suspended in the bath together. They showed that the percentage of strips responding was related to the log of a dose of pituitary put into the bath. By this measurement of "all or nothing" responses Morrell *et al.* obtained more accurate estimations of oxytocic activity than previous workers, but they made no statement of the reliability of each estimate. One estimate of potency had an error of 35 per cent. If this was associated with a high standard error for that particular determination the observer would have been warned that the estimate was unreliable, but it was included in a table of results all of which were considered satisfactory. As Bliss (1941) pointed out, "a determination of potency should always include an estimate of its error, computed as an integral part of the assay." Gaddum (1933) provided the basis for a biological assay which included an estimate of the precision of the result and showed that his formulae applied equally well to measured responses and to quantal data. Schild (1942) published an account of a null hypothesis assay conducted on statistically sound principles. His method has been followed almost completely to obtain the results in this paper.

METHODS

A modification of the classical method of Dale and Laidlaw was adopted.

The rat's uterus was used as test preparation since rats are cheaper and more easily obtained than guinea-pigs. Moreover, Garcia de Jalon, Bayo Bayo, and Garcia de Jalon (1945) have shown that most rats' uteri do not contract spontaneously in Locke's solution in which the calcium and glucose concentrations are $\frac{1}{4}$ and $\frac{1}{2}$ respectively of the usual. One horn of the uterus was suspended in a 10-ml. bath in modified Locke's solution. One end was attached to an

isotonic and linear lever of Schild's (1947) design. The lever was so arranged that the load on the uterus was about 1.2 g. and the contractions were magnified four times. It was equipped with a glass frontal writing point and its angular excursion was limited to 30° each side of the horizontal.

The bath was supplied with oxygen containing 5 per cent CO_2; this was to eliminate possible changes of *p*H, but the CO_2 is probably unnecessary when the Locke's solution is replaced at short intervals.

Non-pregnant white rats were used. Their weights varied from 120 to 200 g. No record was kept of the position in the oestrus cycle of each rat. Table I shows the variation which may be expected within a group of rats.

TABLE I

Assay No.	No. of rats	Spontaneous contractions	Remarks
1, 2, 3, 9, 10	4	No	Satisfactory assays
5, 6, 7	2	Yes, overcome	
4	1	Yes, overcome	Unsatisfactory assays
8	1	Yes	
—	1	Yes	Useless

It will be seen that 6 rats were good, 2 poor, and 1 useless out of a total of 9. In practice the rats in assays 4 and 8 would have been discarded after a short time. The uterus in assay 4 produced only 12 contractions. In assay 8 the uterus had so much spontaneous activity that it was obviously unreliable.

Performance of an assay

1. The rat is killed by a blow on the head and bled out. The uterus is removed and one horn is suspended in the bath and attached to the lever. The temperature must be constant at about 32° C.

2. Doses of pituitary should be given without delay at regular intervals of 3 or 4 min. Two doses must be found such that the contraction for the higher dose is at least twice as great as that for the lower dose. Dose ratios of 4:3, 3:2, 8:5, and 2:1 have been used (4:3 is the most common). In order to ensure linearity between response and log dose it is best to use contractions below 80 per cent of maximal (see *Tests for Linearity* below).

If spontaneous contractions are troublesome they may sometimes be overcome by lowering the temperature or by reducing the time between successive doses. If the uterus is very insensitive (i.e., will not respond to 0.05 unit) the sensitivity may sometimes be increased by raising the temperature.

3. The strength of the unknown must be guessed by matching it with the standard. The error of the assay is much smaller if a good guess is made.

4. The assay is now continued exactly as described by Schild (1942) for histamine assays. Four doses are used, two of standard and two of unknown. The ratio of high dose to low dose should be the same for standard and unknown. Each dose is given once in each group of four doses and its position within the group is decided by chance. Fig. 1 shows the record obtained in one assay. The drum was turned on 15 sec. before the dose was given. The uterus

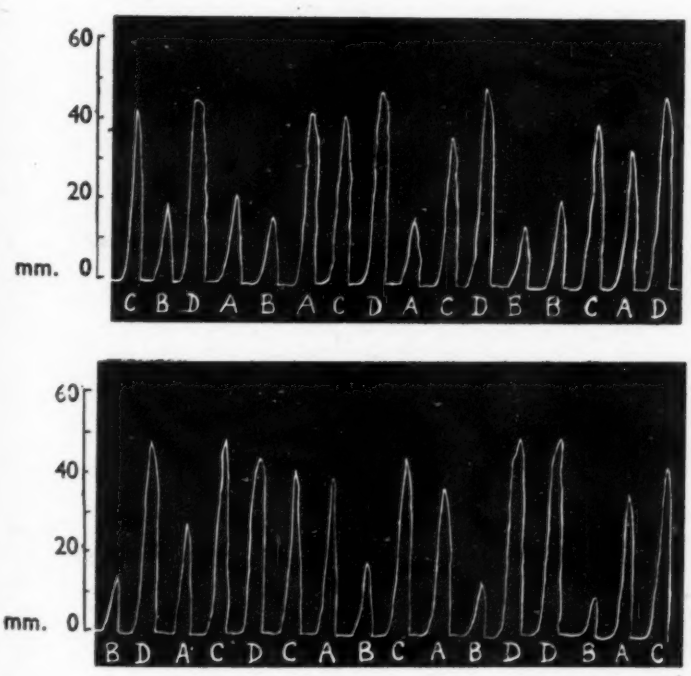

FIG. 1.—An assay of pituitary. The record shows thirty-two contractions of a rat's uterus. The contractions are responses to four different doses of pituitary A, B, C, and D, each of which is given once in each group of 4 contractions. There are eight groups in all. A = 0.05 units, B = 0.04 units, C = 0.064 units, and D = 0.08 units. B and C were treated as "standard." A and D were treated as "unknown." A : D = B : C = 5 : 8. Estimate of unknown: standard = 1.25. True value unknown: standard $= \frac{0.05}{0.04}$ = 1.25. A dose was put into the bath every 4 min. and washed out after 45 sec. Weight of rat 140 g. Temperature 34°–36° C. Load on uterus 1.3 g. Total experimental time 4 hr.

contracted for 45 sec., after which the drum was stopped and the bath was refilled with Locke's solution. A and D were dilutions of the unknown, and B and C were dilutions of the standard; C was stronger than B in the proportion 8 to 5. The ratio of D to A is the same as that of C to B.

TABLE II

Heights of contractions in mm. = y

	Dose units	log dose $= x$	1	2	3	4	5	6	7	8	Sum
D			48	50	52	51	49	45	52	51	398
C	0.064	0.806	43	43	38	42	48	43	44	44	345
A			21	43	17	35	28	39	37	35	255
B	0.04	0.602	21	18	16	25	16	19	14	12	141
Group totals			133	154	123	153	141	146	147	142	1139

RESULTS

The results of this assay are shown in Table II.

In Fig. 2 the mean height of contraction in response to each dose is plotted against the log dose. The "null hypothesis" states that there is no difference between the standard solution and the unknown solution, and so the standard log doses are used in plotting the points for the unknown. Since the unknown and standard contained the same active principle the two lines joining the mean responses to high and low doses of each are parallel. The lines are the regression lines relating height of contraction to log dose, and they were drawn by eye to be the parallel lines that fitted the points most nearly. The regression coefficient b is the slope of each line. An estimate of the potency of the unknown solution can be made by using the two regression lines. The horizontal distance (M) between the lines is the difference between log doses producing the same effect, and it is therefore the log of the ratio of unknown to standard. By elementary geometry

$$M = \frac{\bar{y}_u - \bar{y}_s}{b}$$

where $\bar{y}_u$ and $\bar{y}_s$ are the mean responses to unknown and standard respectively; if b is known M may be calculated.

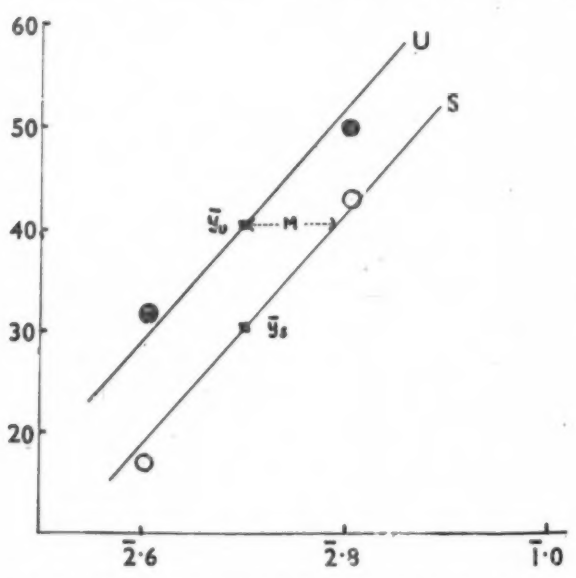

FIG. 2.—Ordinates: mean height of contraction in mm. Abscissae: $\log_{10}$ dose in units of pituitary. White circles are the mean responses for high and low doses of standard, black circles are the mean responses for high and low doses of unknown. The black squares $\bar{y}$ and $\bar{y}_u$ are the means of all the responses to standard and unknown. S and U are the regression lines relating response to log dose of standard and unknown. The lines were drawn by eye. M is the distance between the lines. M = log unknown − log standard = $\log \frac{\text{unknown}}{\text{standard}}$ Graphically $M = 0.09$. $\therefore \frac{\text{unknown}}{\text{standard}}$ = antilog 0.09 = 1.23.

An estimate of potency of a preparation does not give much information about the true potency unless it is accompanied by a statement of its error. The simple graphical measurement of M as described above does not enable us to estimate the reliability of the estimate M; for this we need to calculate the standard error of M. In order to do this M should be calculated rather than determined graphically, and its standard error must also be calculated. The methods of calculation and analysis of variance given by Schild are simple and quick. An example is given below. When the method of assay is slightly varied, for instance, to include three dose levels of standard and of unknown, more complex methods must be used. These may be found in any standard textbook of statistics, but are given particularly clearly by Finney (1947).

Calculation

In order to determine M we must calculate the numerator and denominator of the expression

$$\frac{\bar{y}_u - \bar{y}_s}{b}$$

1. From Table II $\bar{y}_u = \dfrac{398 + 255}{\frac{1}{2}n}$

and $\bar{y}_s = \dfrac{345 + 141}{\frac{1}{2}n}$

where n is the total number of responses; $n = 32$.

2. When only 4 doses are used the equation for b is :

$$b = \frac{\text{sum of responses to high doses} - \text{sum of responses to low doses}}{\frac{1}{2}n \times (\log \text{high dose} - \log \text{low dose})}$$

With the data from Table II

$$b = \frac{(398 + 345) - (255 + 141)}{\frac{1}{2}n \times d}$$

where d = log high dose − log low dose
= log 0.064 − log 0.04
= $\bar{2}.806 - \bar{2}.602$
= 0.204

The value of b will be needed later. It is

$$b = \frac{398 + 345 - 255 - 141}{16 \times 0.204}$$
$$= 106.3$$

For calculation of M, however, it is simpler to use the above expressions, noticing that $\frac{1}{2}n$ occurs in numerator and denominator of the expression for M and so disappears.

$$M = \frac{[(398 + 255) - (345 + 141)] \times 0.204}{(398 + 345) - (255 + 141)}$$
$$= \frac{167 \times 0.204}{347}$$
$$= 0.09811$$

$$\frac{\text{Unknown}}{\text{Standard}} = R = \text{antilog } M = 1.25.$$

Since this assay was conducted with two dilutions, "unknown" and "standard," of the same pituitary extract, the true value of the ratio $\frac{\text{"unknown"}}{\text{standard}} = \rho$ was known. The "unknown" doses of D and A were 0.08 unit and 0.05 unit respectively. Hence

$$\rho = \frac{D}{C} = \frac{A}{B} = \frac{0.08}{0.064} = 1.25$$

In order to make a statement about the reliability of the estimate R, the standard error (s_M) of M must be calculated. This is obtained from the standard deviation (s_y) of a single observation (y). The standard deviation of a single observation—the error of the assay—can only be obtained through an analysis of variance which segregates variation due to known causes from that due to the variability of the preparation.

Table III shows that the sums of squares of deviations attributable to the variation

1. between groups (variation of the preparation with time)

$$= \tfrac{1}{4}(133^2 + 154^2 + 123^2 + \text{etc.}) - \frac{1139^2}{32} = 182$$

2. between standard and unknown

$$= \frac{(398 + 255 - 345 - 141)^2}{32} = 872$$

3. between high doses and low doses (regression)

$$= \frac{(398 + 345 - 255 - 141)^2}{32} = 3763$$

4. between the slopes of the two regression lines (deviations from parallelism)

$$= \frac{(398 + 141 - 345 - 255)^2}{32} = 116$$

The sum of squares of deviations of all the observations from the common mean = total sum of squares

$$= (48^2 + 50^2 + 52^2 + \text{etc.}) - \frac{1139^2}{32}$$
$$= 5536$$

When all the sums of squares due to known causes have been subtracted from the total the remainder is due to the error of the assay. This remainder, divided by its degrees of freedom, gives the variance of a single observation according to the usual formula for standard deviation. The standard deviation of a single observation is the square root of the variance. In the example the residual sum of squares of deviations = 5536 − 182 − 872 − 3763 − 116 = 603. This had 21 degrees of freedom because from the total number (32 − 1) 7 must be subtracted for the 8 different groups and one each for the other three known sources of variation.

$$\text{The variance } s_y^2 = \frac{603}{21} = 28.71$$

TABLE III

Source of variation	Sum of squares	Degrees of freedom	Variance	F	P
1. Groups ..	182	7	26	1.1	>0.05
2. Standard and unknown ..	872	1	872	30	<0.01
3. Regression ..	3763	1	3763	131	<0.01
4. Deviation from parallelism ..	116	1	116	4	>0.05
5. Error ..	603	21	28.71		
Total ..	5536	31			

F is the variance ratio, e.g., for "Deviation from parallelism" $F = \frac{116}{28.7} = 4$. A table of F for $n_1 = 1$ and $n_2 = 21$ (where n_1, n_2 are the degrees of freedom for "Deviations from parallelism" and for "Error" variance respectively) gives $P > 0.05$. This means that there is a proba-

bility of more than 5 per cent that this deviation from parallelism would occur by chance and so the slopes of the two regression lines are not significantly different. The values given in the column P are the probabilities that such variation would occur by chance. Thus there is less than a one per cent probability that so great a variation between standard and unknown would have occurred had the unknown not been different from the standard. The unknown is said to be significantly different from the standard. Similarly, the regression is significantly different from zero. The variation between groups is not greater than would be expected by chance in more than one in twenty experiments. (Here $F = \frac{28{\cdot}7}{26} = 1.1$ because $F = \frac{\text{larger mean square}}{\text{smaller mean square}}$ and hence $n_1 = 21$ and $n_2 = 7$).

The standard error of M

The standard error of the estimate of M may be obtained from Schild's formula:

$$s_M^2 = \frac{4s_y^2}{nb^2}\left(\frac{M^2}{d^2} + 1\right)$$

In the example

$$s_M^2 = \frac{4 \times 28.71}{32 \times (106.3)^2}\left[\frac{(0.09811)^2}{(0.2041)^2} + 1\right]$$

$$= \frac{114.84 \times 1.2312}{361600}$$

$$= 0.0003909$$

$$\therefore s_M = \sqrt{0.0003909} = 0.01976$$

The fiducial limits of M

The limits of the value of M are obtained from adding and subtracting the standard error of M multiplied by t. The value of t depends on the degrees of freedom associated with the error sum of squares. In the example there were 21 degrees of freedom, and so t is obtained from the table under $n = 21$. For a probability level of 0.05, $t_{21} = 2.08$.

$$s_M \times 2.080 = 0.0421$$

$$\therefore M \pm s_M \times 2.080 = 0.0981 \pm 0.0421$$

Hence the fiducial limits ($P = 0.05$) for M are

0.0560 and 0.1402

The limits for R are :

R_1 = antilog 0.0560 = 1.14

R_2 = antilog 0.1402 = 1.38

That is to say, there is only one chance in twenty that these results would have been obtained if the true value of the unknown lay outside the limits 1.14 – 1.38.

Now $1.38 - 1.14 = 0.24$ and $\frac{0{\cdot}24}{2} = 0.12$

$\therefore$ the approximate range of results is

$$R \pm 0.12 = 1.25 \pm 0.12$$

Hence the limit of error is $\frac{0.12}{1.25} \times 100 = 9.6\%$.

(This assumes that R_1 and R_2 are equidistant from R, which is not strictly true since M_1 and M_2 are equidistant from M, which is on a logarithmic scale, but the assumption is true enough for an approximate calculation of the limits of error.)

TABLE IV

Assay	Time	n	ρ	R	R_1	R_2	E	s_y/b
1	5	36	1.25	1.22	0.97	1.55	2.6	0.145
2	4½	32	1.25	1.25	1.14	1.38	0	0.050
3	5	32	1.14	1.10	1.06	1.13	3.6	0.018
4	2	12	0.80	0.91	0.81	1.02	14	0.044
5	4	24	1.08	1.10	0.96	1.25	1.9	0.062
6	3½	24	1.07	1.09	1.02	1.18	1.25	0.034
7	3	24	1.07	1.05	0.96	1.15	1.25	0.044
8	8	24(-4)	0.91	1.06	0.77	1.56	15	0.131
9	2½	20	1.00	0.99	0.94	1.04	0.85	0.022
10	2	20	1.07	1.01	0.96	1.06	5.8	0.022

Table IV includes all of a recent series of 10 assays in order to show the variation which may be expected from a group of 9 rats. Eight out of these 10 assays were considered satisfactory. Two assays (4 and 8) were unsatisfactory and in practice the results from them would have been subjected to further tests. The uterus gave only 12 contractions in assay 4 and then lost all sensitivity. In assay 8 the uterus had a great deal of spontaneous activity and the contractions were obviously unreliable. The assays which are bracketed were performed on the two horns of the uterus from the same rat.

In assays 4–10 the true value of the ratio of unknown to standard (ρ) was unknown to the observer until after the results were obtained. The columns R, R_1 and R_2 show the estimate of ρ and the two fiducial limits (R_1 and R_2) calculated as described above for $P = 0.05$. It will be noticed that ρ falls within the range $R_1 - R_2$ except in assays 3, 4, and 10, where it is just outside the range. Recalculation of R_1 and R_2 according to Schild's equation for the exact limits, which is given in a footnote to his paper, did not alter the values. It must be borne in mind, however, that the statistical analysis is based on the assumption that all measurements are absolutely

accurate and the calculation of error does not include unavoidable errors in pipetting which occur during the dilution of the test solutions. Hence, in order to make a reliable statement about the potency of an unknown, the fiducial limits should be extended by 4 per cent (this allows for 1 per cent error for each dilution). The figures in the column "Time" are the times in hours required for the experimental part of each assay and E is the actual percentage error in the estimate of potency.

The ratio of the standard deviation of a single response to the slope of the regression line is shown in the last column of Table IV (s_y/b). It is an inverse measure of the usefulness of the preparation and for a satisfactory assay (s_y/b) should not be much greater than 0.05.

Gaddum's calculation

Gaddum (1938) calculated the standard error of pituitary assays performed by the method of Dale and Laidlaw. He used only those assays which were considered satisfactory and obtained a value for the standard error of 7.73 per cent, which gave fiducial limits at 19.9 per cent ($P = 0.01$). If his calculation is repeated with the results of the assays in Table IV, excluding assays 4 and 8 for reasons given above and using ρ instead of his mean estimated potency, the standard error is 2.84 per cent and fiducial limits are at 9.4 per cent (for $P=0.01$ $t_7=3.499$), which is about half the value found by Gaddum for the original Dale and Laidlaw method.

The dose-response relationship

The calculations and statistical analysis of the results are based on the assumption that the relation between response and log dose is exactly linear. In fact, the curve relating response to log dose is sigmoid and approximates to linearity over part of the response range. Clearly it should be the aim of the observer to confine the responses of the uterus to this linear part of the range. Since the region of linearity varies from one preparation to another it must be left to the observer to decide its position, but it has been found that for most uteri any responses less than 80 per cent of the maximal can be used.

Tests for linearity

Assay No. 1 was exceptional in that 6 different doses (3 standard and 3 unknown) were used. Here a test of linearity was possible without knowing the true value of the "unknown." It showed that there was no significant departure from linearity. The calculations for this assay were slightly more complex than for the 4-dose assays and were performed as described by Finney (1947). The 6-dose design is not recommended for routine work because the experimental procedure is more complicated and the time interval between successive doses may be too short for the additional diluting needed for 6 doses.

It should be realized, however, that the 4-dose design suffers from the defect that no test of linearity can be applied from the data of a single assay. The worker is safeguarded to a certain extent by the test for parallelism. The variance due to deviations from parallelism would be significant if there were any serious departure from linearity and if M_2 were not negligible.

Table V shows the variance ratios (F) for deviations from parallelism. An F value smaller than that for $P = 0.05$ indicates that the deviation was not significant. In assay 9 the deviation from parallelism was just significant. In this assay the strengths of the unknown and standard were exactly the same owing to a lucky initial guess of the potency. Hence it was not possible to calculate the deviation from linearity as was done by Schild. However, the estimate of the potency of the "unknown" in assay 9 was a good one, which is in accordance with Schild's conclusion that the method is relatively insensitive to deviations from parallelism. The last two columns in Table V show the highest and the lowest contractions as recorded on the drum. The maximum contraction was generally not recorded, but general experience with the rat uterus preparation indicates that most uteri are capable of 100 mm. contraction with the same apparatus, and so the figures in the table are approximate percentages of the maximum.

TABLE V

Assay	Deviation from parallelism F	$F(P=0.05)$	$F(P=0.01)$	Highest contraction mm.	Lowest contraction mm.
1	26	249		65	2
2	14	244		52	16
3	10	244		73	3
4	34	249		61	3
5	10	244		32	15
6	1.5	244		35	11
7	2	4.3		49	1
8	1.5	4.75		85	0
9	5.2	4.75	9.33	80	17
10	50	244		96	11

Summary

1. Schild's null hypothesis method was applied to the assay of posterior pituitary extract according to Dale and Laidlaw.

2. A rat's uterus was used as the test preparation.

3. One assay is described and the result is calculated by Schild's method.

4. Six rats out of a total of nine could be used for satisfactory assays.

5. Eight satisfactory assays were performed. The mean percentage error was 2.16. Fiducial limits for the estimate of potency were calculated. The mean experimental time of an assay was 3¼ hours.

This work was done at the suggestion of Prof. J. H. Burn. My thanks are due to Dr. D. J. Finney and his staff for help in the calculations.

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SULPHYDRYL ADDITION COMPOUNDS OF SOME QUINONES AND RELATED SUBSTANCES IN THEIR ACTION ON THE GROWTH OF NORMAL CELLS

BY

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We have reported recently (1948) that maleic acid produces in tissue cultures of chick fibroblasts a mitotic inhibition of the same kind and of the same order as 2-methyl-1:4-naphthohydroquinone diphosphate, studied by J. S. Mitchell and I. Simon-Reuss (1947) with the same biological material. This similarity is easily understood if the substituted hydroquinone is degraded in the dividing cell to a quinone, as the quinone molecule contains in its aliphatic part the residue of maleic acid. The question therefore arises whether maleic acid and the quinones have properties in common which may help us to understand the similarity of their antimitotic activity.

Maleic acid and the quinones excel by the ease with which they add other molecules. Amongst the substances which are added the sulphydryl compounds play a prominent part, physiologically as well as chemically. Their role in cell division is well established by the investigations of Shearer (1922), Hammet (1930), Rapkine (1931), Ephrussi (1931), Chalkley (1937), Brachet (1940), and many others. Chemically it has been shown that maleic acid adds thiolacetic acid, cysteine, and glutathione to give well-defined products. Furthermore the reactivity of maleic acid towards –SH compounds permits its use as an inhibitor of –SH enzymes (Morgan and Friedmann, 1938a, b, and c). On the basis of these results the retardation of malignant growth by maleic acid (Brunschwig *et al.*, 1946) has been discussed. The fixation of –SH-containing enzymes by quinone has been suggested by Potter (1942) as an explanation of the growth-inhibiting action of azo dyes. In the naphthoquinone series Fieser and Fieser (1944) emphasize the smooth addition of –SH compounds by naphthoquinones in connection with their physiological activity. Colwell and McCall (1945) suggest that the mode of antibacterial action of 2-methyl-1:4-naphthoquinone is a blocking of essential enzymes or essential bacterial metabolites by its combination with sulphydryl groups.

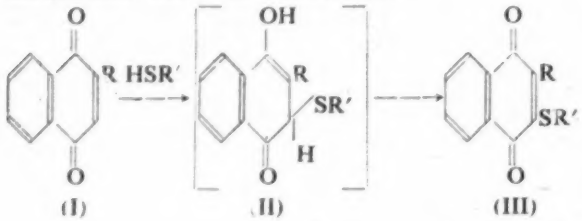
In our own experiments (1948) a parallelism between mitotic inhibition and –SH uptake is apparent. Thus maleic acid adds –SH compounds: it is a strong antimitotic. The *trans*-isomer, fumaric acid, and the methyl derivatives, citraconic acid and mesaconic acid, show no –SH uptake: they are devoid of antimitotic activity. Naphthoquinone and its 2-methyl derivative easily add –SH compounds: they are both strongly antimitotic.

The parallelism between mitotic inhibition and –SH uptake, manifested by maleic acid and the quinones, has been used by us as a starting point for an experimental approach to the underlying problems.

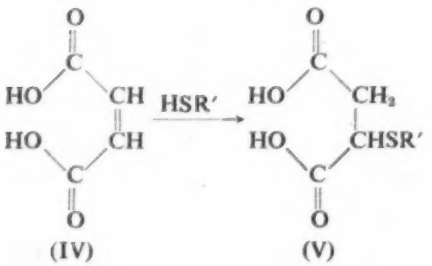
As in tissue cultures the diphosphates of the hydroquinones and not the free quinones were applied, a comparison between maleic acid and the quinones presupposes that the quinones have the same degree of antimitotic activity as the hydroquinones. Lehmann (1942) has shown that this is so. Nevertheless we are fully aware that our choice is an arbitrary one. It compels us to set aside the processes which lead from the diphosphates of hydroquinones to the quinones, reactions which may be associated with physiological processes as important as, if not more important than, those connected with the quinone structure.

The naphthoquinones (I) and maleic acid (IV) behave somewhat differently with –SH compounds: maleic acid forms saturated thio-ethers

of succinic acid (V), but the corresponding naphthalene derivatives (II) are so easily oxidized by unchanged quinone that only thio-ethers of naphthoquinone (III) can be isolated.



(I) (II) (III)



(IV) (V)

Addition products of type (III), where R = H or CH_3, have been prepared from the quinone and thiolacetic acid and glutathione. Addition products of type (V) have been prepared from maleic acid and thiolacetic acid, glutathione and cysteine. The action of these substances on the growth of normal cells has been investigated. The present communication gives the results obtained so far.

Experimental

The experiments were carried out on tissue cultures of chick fibroblasts. The technique used has been described in our first paper (1948), to which we refer for details.

The values for mitotic inhibition and phase distribution obtained with the different substances are collected in the Table. The cytological description will be given by Mrs. I. Simon-Reuss in another paper.

1. S–(1 : 4-naphthoquinonyl-2)-thiolacetic acid (III ; R = H, R′ = .S.CH_2CO_2H)

The Table shows that the addition of thiolacetic acid to 1 : 4-naphthoquinone has abolished the strong antimitotic properties of 1 : 4-naphthoquinone. The phase distribution has also become normal. No abnormal mitoses have been observed.

TABLE

Tissue culture: chicken fibroblasts, hanging drop method, 4th passage, 24 hr. cultures, fixed in Susa, stained in Heidenhain's haematoxylin

Exp.	Molar conc.	Mitoses as % of mitoses of controls	Per ent inhibition	Phase distribution in % of mitoses			
				Prophase	Metaphase	Anaphase	Telophase
S-(1 : 4-NAPHTHOQUINONYL-2)-THIOLACETIC ACID (5210 mitotic cells investigated).							
1.	Controls	—	—	14.0	36.9	8.9	40.2
2.	1×10^{-6}	94.6 ± 5.4%	—	14.0	43.1	1.2	36.7
3.	3×10^{-6}	102.8 ± 9.1%	—	13.7	41.6	7.2	37.5
4.	5×10^{-6}	98.6 ± 8.2%	—	14.5	42.3	4.5	38.7
5.	Controls	—	—	14.4	27.9	1.7	56.0
6.	4×10^{-6}	99.3 ± 2.4%	—	15.2	45.1	2.6	37.1
7.	6×10^{-6}	99.6 ± 2.5%	—	15.6	43.6	2.5	38.2
S-(1 : 4-NAPHTHOQUINONYL-2)-GLUTATHIONE (9390 mitotic cells investigated).							
1.	Controls	—	—	16.3	39.2	7.0	37.5
2.	2×10^{-6}	99.3 ± 7.8%	—	17.6	36.5	8.1	37.8
3.	4×10^{-6}	100.9 ± 6.4%	—	17.7	38.8	7.9	35.6
4.	6×10^{-6}	99.8 ± 7.7%	—	17.4	38.9	7.6	36.1
5.	Controls	—	—	18.9	22.1	6.0	53.0
6.	2×10^{-6}	99.8 ± 8.6%	—	11.7	26.3	4.3	57.7
7.	4×10^{-6}	99.8 ± 5.3%	—	16.0	24.1	5.6	53.8
8.	6×10^{-6}	99.0 ± 4.5%	—	13.4	26.1	5.3	55.2
S-(2-METHYL-1 : 4-NAPHTHOQUINONYL-3)-THIOLACETIC ACID (5545 mitotic cells investigated).							
1.	Controls	—	—	17.5	27.0	6.6	48.9
2.	1×10^{-6}	92.1 ± 3.1%	—	17.2	28.1	5.9	48.8
3.	3×10^{-6}	99.7 ± 6.0%	—	14.4	32.8	5.4	47.4
4.	5×10^{-6}	110.5 ± 9.8%	—	13.9	32.4	5.2	48.6
5.	Controls	—	—	14.0	36.9	8.9	40.2
6.	4×10^{-6}	101.4 ± 2.9%	—	17.7	42.9	3.7	35.7
7.	6×10^{-6}	100.7 ± 2.5%	—	16.5	40.0	3.1	40.4

TABLE (*continued*)

Exp.	Molar conc.	Mitoses as % of mitoses of controls	Per cent inhibition	Phase distribution in % of mitoses			
				Prophase	Metaphase	Anaphase	Telophase
S-(2-METHYL-1 : 4-NAPHTHOQUINONYL-3)-GLUTATHIONE (6291 mitotic cells investigated).							
1.	Controls	—	—	17.4	34.5	4.9	43.2
2.	1×10^{-7}	—	—	9.1	62.3	10.0	18.5
3.	3×10^{-7}	—	—	8.4	60.5	9.5	21.7
4.	5×10^{-7}	89.9 ± 3.9%	10.1	17.2	46.0	6.8	30.0
5.	Controls	—	—	18.0	35.0	1.7	45.3
2.	2×10^{-6}	44.1 ± 3.0%	55.9	12.8	36.9	4.1	46.2
4.	4×10^{-6}	47.0 ± 1.5%	53.0	17.1	38.5	8.4	35.9
6.	6×10^{-6}	42.6 ± 2.0%	57.4	12.5	33.9	6.8	46.8
7.	Controls	—	—	18.3	36.5	5.8	39.4
8.	2×10^{-6}	44.5 ± 4.0%	55.5	18.1	33.5	16.0	32.3
9.	4×10^{-6}	41.2 ± 5.8%	58.8	14.7	48.0	3.6	33.7
10.	6×10^{-6}	44.3 ± 3.4%	55.7	11.6	49.2	12.1	27.1
S-THIOLACETO-SUCCINIC ACID (5539 mitotic cells investigated).							
1.	Controls	—	—	20.1	36.4	2.6	40.9
2.	1×10^{-6}	93.9 ± 7.7%	6.0	17.9	38.4	2.0	41.6
3.	3×10^{-6}	71.5 ± 4.8%	28.5	19.7	35.6	3.9	40.8
4.	5×10^{-6}	84.5 ± 6.8%	15.5	22.5	38.6	5.6	33.3
5.	Controls	—	—	17.5	28.2	2.6	51.6
6.	2×10^{-6}	96.0 ± 2.8%	4.0	23.1	21.2	3.1	52.5
7.	4×10^{-6}	70.8 ± 2.4%	29.2	19.0	26.3	2.3	52.4
8.	6×10^{-6}	66.3 ± 3.3%	33.7	18.3	21.9	0.9	58.9
S-CYSTEINO-SUCCINIC ACID (5081 mitotic cells investigated).							
1.	Controls	—	—	22.3	31.3	1.3	45.1
2.	1×10^{-6}	84.7 ± 5.3%	15.3	19.9	32.8	0.6	46.7
3.	3×10^{-6}	81.3 ± 5.4%	18.7	19.6	33.7	0.5	46.2
4.	5×10^{-6}	54.3 ± 3.4%	45.7	14.1	29.8	1.7	54.3
5.	Controls	—	—	19.8	23.0	2.2	55.0
6.	2×10^{-6}	68.3 ± 7.1%	31.7	20.7	21.7	2.5	55.1
7.	4×10^{-6}	68.3 ± 5.9%	31.7	21.4	24.2	3.1	51.4
8.	6×10^{-6}	37.5 ± 3.1%	62.5	19.4	16.5	2.0	62.1
S-GLUTATHIONO-SUCCINIC ACID (7964 mitotic cells investigated).							
1.	Controls	—	—	20.4	19.2	8.4	52.0
2.	1×10^{-6}	86.0 ± 4.8%	14.0	16.1	25.2	9.0	49.7
3.	3×10^{-6}	73.5 ± 5.5%	26.5	16.6	29.1	17.8	36.5
4.	5×10^{-6}	54.9 ± 4.3%	45.3	13.3	35.8	8.1	42.8
5.	Controls	—	—	14.9	34.9	4.6	45.6
6.	2×10^{-6}	70.1 ± 6.6%	29.9	20.7	40.9	11.9	26.7
7.	4×10^{-6}	65.9 ± 6.5%	34.1	16.0	40.6	7.2	36.1
8.	6×10^{-6}	55.7 ± 6.1%	44.8	18.9	28.0	5.3	47.6

2. S–(1 : 4-naphthoquinonyl-2)-glutathione (III ; R=H, R′=.SG)

The substance gives no mitotic inhibition ; the phase distribution is normal. Abnormal cells have not been found.

3. S–(2-methyl-1 : 4-naphthoquinonyl-3)-thiolacetic acid (III ; R=CH_3, R′=$S.CH_2CO_2H$)

Mitoses and phase distribution are normal.

4. S–(2-methyl - 1 : 4 - naphthoquinonyl-3) - gluta - thione (III ; R=CH_3, R′=SG)

The addition of glutathione to 2-methyl-1 : 4-naphthoquinone gives a product which has definite antimitotic properties. Mitotic inhibition seems to start at 5×10^{-7} *M* with inhibition of 10 per cent. The mitotic inhibition increases with rising concentration: at 2×10^{-6} *M* it is 56 per cent. This value seems to represent the maximum of mitotic inhibition, as further increases in concentration are not followed by greater mitotic inhibition. The phase distribution shows features of interest. Disturbances in phase distribution have been observed at 1×10^{-7} and at 3×10^{-7} *M*, i.e. at concentrations where no mitotic inhibitions are found in the 24-hour tissue cultures. Here phase disturbances precede mitotic inhibition. In contrast, no phase

disturbances are to be seen in the 24-hour cultures when the mitotic inhibition reaches its peak. The phase disturbances consist in an accumulation of metaphases and anaphases and a loss of telophases

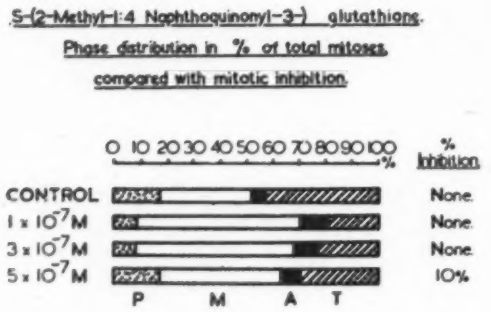

S-(2-Methyl-1:4 Naphthoquinonyl-3-) glutathione.
Phase distribution in % of total mitoses,
compared with mitotic inhibition.
0 10 20 30 40 50 60 70 80 90 100 %
% Inhibition
CONTROL None.
1 x 10⁻⁷M None.
3 x 10⁻⁷M None.
5 x 10⁻⁷M 10%
P M A T

FIG. 1.—Disturbances in phase distribution preceding mitotic inhibition.

(Fig. 1). Abnormal mitoses are present in the whole range of concentrations investigated, even at 1×10^{-7} and 3×10^{-7} *M*.

5. S–thiolaceto-succinic acid (V ; R = $S.CH_2CO_2H$)

The substance has weak antimitotic properties. At 6×10^{-6} *M* the mitotic inhibition is only 33.7 per cent. The phase distribution is apparently not disturbed. Abnormal mitoses are present in all concentrations investigated.

6. S–cysteino-succinic acid (V ; R = $S.CH_2CH(NH_2)CO_2H$)

The mitotic inhibition of S–cysteino-succinic acid is more pronounced than the inhibition by

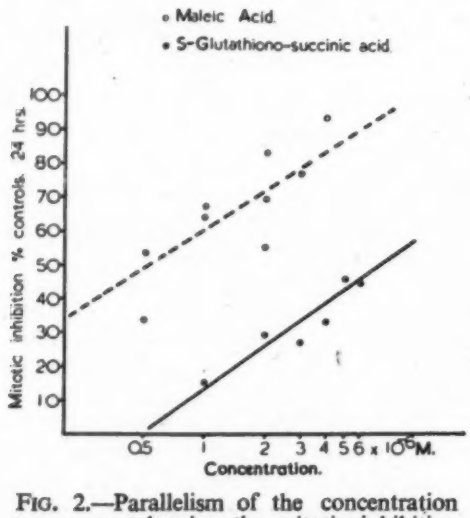


FIG. 2.—Parallelism of the concentration curves showing the mitotic inhibition of maleic acid and of S-glutathiono-succinic acid.

substance 5. At 6×10^{-6} *M* an inhibition of 62.5 per cent has been found. The phase distribution is normal. A few abnormal cells are present at all concentrations. The small outgrowth of the cultures at the higher concentrations (5 and 6×10^{-6} *M*) has to be mentioned.

7. S–glutathiono-succinic acid (V ; R = SG)

The mitotic inhibition of S–glutathiono-succinic acid is weaker than the inhibition produced by the cysteine adduct (substance 6). The inhibition increases with rising concentrations. The concentration curve follows a logarithmic line when plotted against the $\log_{10}$ of the concentrations. The graphic comparison with the corresponding line representing the concentration curve of maleic acid shows that the two lines take parallel courses (Fig. 2). The phase distribution shows accumulation of metaphases and irregular anaphases ; abnormal mitoses are to be seen at all concentrations.

DISCUSSION

The experiments carried out with sulphydryl addition compounds of some quinones and of maleic acid, described in this paper, deal with two groups of adducts which are different in the part of the molecule, apparently connected with their physiological activity. The quinones have given unsaturated addition products (III) which can be regarded as derived from maleic acid, whilst the maleic acid adducts are saturated succinic acid derivatives (V).

Mitotic inhibition has been found in both groups. Unsaturation of the adducts, therefore, does not seem to be the decisive factor determining their mitotic activities. This result can be supported by an interesting analogy: Lettré and Mohn (1946) have found that the dihydro-derivatives of diethyl stilboestrol have antimitotic properties as well as the unsaturated diethyl stilboestrol. On the other hand, v. Möllendorff's observations (1941) on male sex hormones show clearly that in other groups the unsaturation of the molecule is essential. Only the unsaturated members of this group develop antimitotic properties, whilst the activity as male sex hormones is displayed by the saturated compounds as well. The possible physiological transition of the substances investigated by us from saturation to unsaturation or conversely must be kept in mind for further investigations.

The addition of SH compounds to maleic acid results in the formation of a centre of asymmetry in the adduct when thiolacetic acid is used for this reaction, or of an additional centre when cysteine or glutathione are used. We intend to

supplement our investigations by resolving S–thiolaceto–succinic acid into its optical isomers and investigating their antimitotic activities. S–cysteino–succinic acid seems to be formed under the stereochemically directing influence of L–cysteine (Morgan and Friedmann, 1938c).

The results obtained with the sulphydryl addition compounds of 2-methyl-1:4-naphthoquinone are interesting in several respects. The S–thiolaceto-derivative was completely inactive as an inhibitor of mitosis, whilst the glutathione derivative was active. Here, clearly, the importance of the sulphydryl-carrying reactant comes to light. It has been stressed from the beginning in the maleic acid series that the reactivity of the sulphydryl group with the activated double bond is different from case to case. In the quinone series the interesting results of Walsh and Walsh (1948), showing that liver hexosediphosphatase exhibited a much greater sensitivity towards quinones than did the other phosphatases, point in the same direction.

The activity of the glutathione derivative of 2-methyl-1:4-naphthoquinone as an inhibitor of mitosis, compared with the lack of antimitotic activity of the methyl free S– (1:4-naphthoquinolyl-2)-glutathione, shows a new feature of the methyl group. So far we have seen that the introduction of a methyl group in the quinones as well as in maleic acid has been followed by a decrease of their antimitotic activity. Now the elimination of the methyl group leads to a product which has lost its antimitotic activity. Chemical analogy shows that the 2-methyl group exerts a distinct retarding effect in the introduction of substituents at position 3 by way of 1:4 – addition reactions (Fieser and Fieser, 1944, pp. 737 and 743), but there are exceptions where the presence of a 2-methyl group increases the reactivity.

The adducts of thiolacetic acid and of glutathione to 1:4-naphthoquinone were both inactive as antimitotic agents. Other sulphydryl substituents have not been investigated. The different biological results obtained by adding thiolacetic acid or glutathione to 2-methyl-1:4-naphthoquinone show that the possibility cannot be dismissed that other –SH addition products to 1:4-naphthoquinone may display antimitotic activity.

The substances formed by the addition of thiolacetic acid to some quinones are acids in which the sulphur is linked ether-fashion. If one replaces the sulphur by oxygen one comes in the aromatic series to substances related to phenoxyacetic acid. These are known to be differential growth-inhibitors for plants. It is claimed that their action is similar to that of colchicine (Arvy and Lhoste, 1946).

The examination of the reaction products of quinones and maleic acid with –SH compounds has been undertaken in order to see whether their action on mitoses would allow us to explain the parallelism between mitotic inhibition and –SH uptake, established experimentally. The present investigation affords no clear solution of this problem. From the seven adducts examined three have exerted no mitotic inhibition (1, 2, and 3) and four were active as mitotic inhibitors (substances 4, 5, 6, and 7). Dealing with the same problem, Michael (1948) has found unimpaired physiological activity with the quinonoid fuscin after addition of thiolacetic acid. On the other hand Kuhn and Beinert (1945), who investigated the inhibition of carboxylase, have shown that S-cysteino-*p*-benzoquinone, resulting from the addition of cysteine to *p*-benzoquinone, has lost the strong inhibitory activity of *p*-benzoquinone. Furthermore, 2-methyl substitution in 1:4-naphthoquinone decreases the antimitotic activity, whereas the introduction of a methyl group in the *ortho* position in –S–derivatives of 1:4-naphthoquinone increases the antimitotic activity of the new compound as shown in this paper.

Simple thiol addition compounds like those we have prepared and investigated may play no part in the mitotic inhibition induced by quinones or maleic acid. No satisfactory evidence is available to discuss other possibilities.

Chemical Section

1. *S-(1 : 4-naphthoquinonyl-2)-thiolacetic acid*

The substance was prepared following the directions given by Fieser and Turner (1947) for the preparation of S-(2-methyl-1:4-naphthoquinonyl-3)-thiolacetic acid. 1:4-Naphthoquinone (2.6 g.) was dissolved in warm alcohol (120 c.c.), cooled to room temperature, and mixed with a solution of thiolacetic acid (1.5 g., 1.13 c.c., 1 mol. quantity) in alcohol (4 c.c.). After standing overnight the dark solution was evaporated *in vacuo*, when yellow plates appeared.

Yield 0.8 g., m.p. 173–5° (decomp.) after softening at 164° (20 per cent yield of crude product). A portion (0.5 g.) was dissolved in a solution of sodium bicarbonate (0.5 g.) in water (25 c.c.) and well extracted with ether. The aqueous phase was acidified to Congo red with 5*N* sulphuric acid and the precipitated yellow gel was extracted with a large volume (500 c.c. in all) of ether. The extract was washed twice with water, dried over sodium sulphate, and evaporated to dryness. The crystalline residue was recrystallized from alcohol giving 0.35 g. yellow plates m.p. 183.5° (decomp.). For analysis, the *compound* was purified on a column of alumina, when

washing with methanol removed traces of a dark-coloured impurity. *S-(1 : 4-naphthoquinonyl-2)-thiol-acetic acid* was eluted from the column with aqueous bicarbonate, isolated after acidification and recrystallized twice from alcohol to m.p. 183.5° (decomp.). Found (in material dried at 80° *in vacuo*): C, 58.0; H, 3.4. $C_{12}H_8O_4S$ requires C, 58.1; H, 3.3 per cent.

2. *S-(1 : 4-naphthoquinonyl-2)-glutathione*

The method described by Fieser and Fieser (1944) was used for the preparation of this substance. 1:4-Naphthoquinone (316 mg., 1/500 mol.) was dissolved in warm alcohol (10 c.c.) and cooled to room temperature. The solution was added at once to a solution of glutathione (307 mg., 1/1,000 mol.) in water (4 c.c.) and alcohol (6 c.c.). An immediate darkening occurred with the separation of a yellow solid, which was left overnight at room temperature, filtered off, well washed with alcohol, and dried.

Yield 440 mg. yellow amorphous solid (95 per cent yield). Found (in material dried *in vacuo* at room temperature): C, 51.6; H, 4.9; N, 9.2. $C_{20}H_{21}O_8N_3S$ requires C, 51.8; H, 4.6; N, 9.1 per cent.

3. *S-(2-methyl-1 : 4-naphthoquinonyl-3)-thiolacetic acid*

Prepared by the method of Fieser and Turner (1947), except that after reduction by aqueous hydrosulphite it was found more convenient to re-oxidize to the quinone by means of 10 per cent aqueous ferric chloride containing 1/5 of its volume of 5*N* sulphuric acid.

4. *S-(2-methyl-1 : 4-naphthoquinonyl-3)-glutathione*

Prepared by the method of Fieser and Fieser (1944) in a yield of 86 per cent.

5, 6, *and* 7. *S-thiolaceto-succinic acid* (5), *S-cysteino-succinic acid* (6), *and S-glutathiono-succinic acid* (7) have been prepared by Morgan and Friedmann (1938a). The original substances analysed by these authors have been used for our experiments.

SUMMARY

1. Evidence is given that the mitotic inhibition produced by some quinones and by maleic acid goes parallel with the –SH uptake of these substances.

2. S–(1 : 4-naphthoquinonyl-2)-thiolacetic acid (1), S–(1 : 4-naphthoquinonyl-2)-glutathione (2), S–(2-methyl-1 : 4-naphthoquinonyl-3)-thiol-acetic acid (3), S–(2-methyl-1 : 4-naphthoquinonyl-3)-glutathione (4), S–thiolaceto-succinic acid (5), S–cysteino-succinic acid (6), S–glutathiono-succinic acid (7), have been prepared and investigated in tissue cultures of chick fibroblasts. Substances (1), (2), and (3) give no mitotic inhibition, substances (4), (5), (6), and (7) were inhibitors of mitosis.

3. The biological results are discussed.

4. Simple thiol addition compounds to quinones and maleic acid like those mentioned above seem to play no part in the mitotic inhibition induced by some quinones and by maleic acid.

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THE ACTION OF CHOLINE DERIVATIVES ON ISOLATED RABBIT AURICLES WHEN ARRESTED BY PALUDRINE

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The observation made by Sachs (1937), that minute doses of acetylcholine would stimulate the beat of isolated hearts, has now been repeated by many workers (Spadolini and Domini, 1940; Rothberger and Sachs, 1938; McDowall, 1946; Haney and Lindgren, 1945). Of these Spadolini and Domini were the first to postulate that this action was due to the liberation of an adrenaline-like substance from either ganglia or chromaffin tissue in the heart. Hoffmann *et al.* (1945) and McNamara, Krop, and McKay (1948) confirmed that an adrenaline-like substance is liberated from the heart muscle by acetylcholine.

In a previous paper from this laboratory (Burn and Vane, 1948), it was shown that if isolated rabbit's auricles were exposed to the action of paludrine, then the inhibitory effect of acetylcholine was gradually changed to a stimulation. This change was accompanied by a gradual reduction in the rate and amplitude of beat. Within 7–38 min. after the paludrine had been added the beat of the auricles stopped, usually abruptly, and the auricles remained quiescent even when left in fresh Ringer-Locke solution for periods of up to an hour. During this time acetylcholine restarted the beat. These observations have now been extended to include other acetylcholine-like compounds and adrenaline.

RESULTS

The auricles of a freshly killed rabbit were dissected clean of fat and ventricular tissue; they were then suspended in well-oxygenated Ringer-Locke solution at 29° C., so that the contractions were recorded on smoked paper by a light straw lever, an upstroke representing systole.

Acetyl-β-methylcholine.—Fig. 1*a* shows the normal inhibitory effect of acetylcholine and acetyl-β-methylcholine on the beat. Paludrine (8 mg.) was added to the bath of 75 ml. Ringer-Locke solution. The amplitude and rate of beat steadily declined until they were about 60 per cent of the

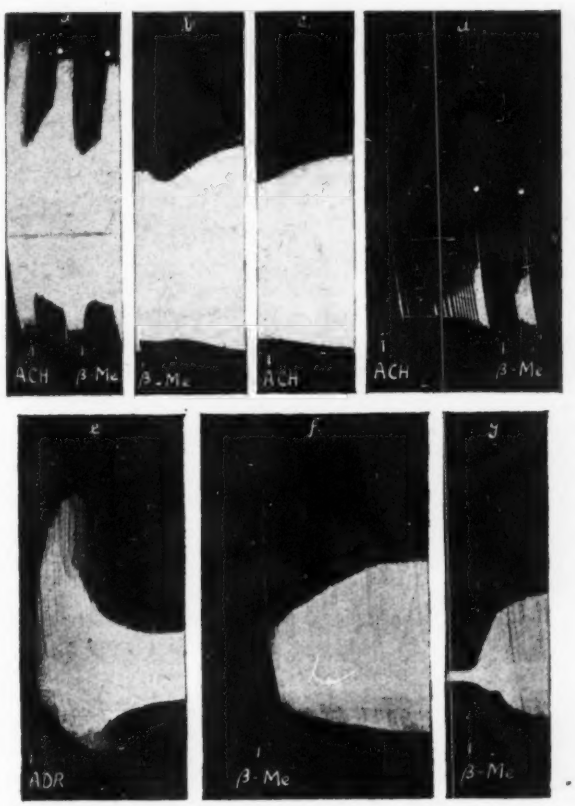

FIG. 1.—Isolated rabbit auricles. (*a*) Acetylcholine (40 μg.). Acetyl-β-methylcholine (20 μg.). Between (*a*) and (*b*) paludrine (8 mg.). (*b*) Acetyl-β-methylcholine (40 μg.) and (*c*) Acetylcholine (40 μg.). Both caused stimulation. The beat stopped. (*d*) Beat restarted with acetylcholine (200 μg.). Stopped when washed out. Restarted with acetyl-β-methylcholine (200 μg.). Stopped when washed out. (*e*) Restarted with adrenaline (20 μg.). Irregular beat, which stopped when washed out. (*f*) Restarted with acetyl-β-methylcholine (100 μg.). Continued beating regularly and with larger amplitude than when started with adrenaline. (*g*) Stimulated by acetyl-β-methylcholine (100 μg.). Dots above record indicate that the bath was changed to fresh Ringer-Locke solution.

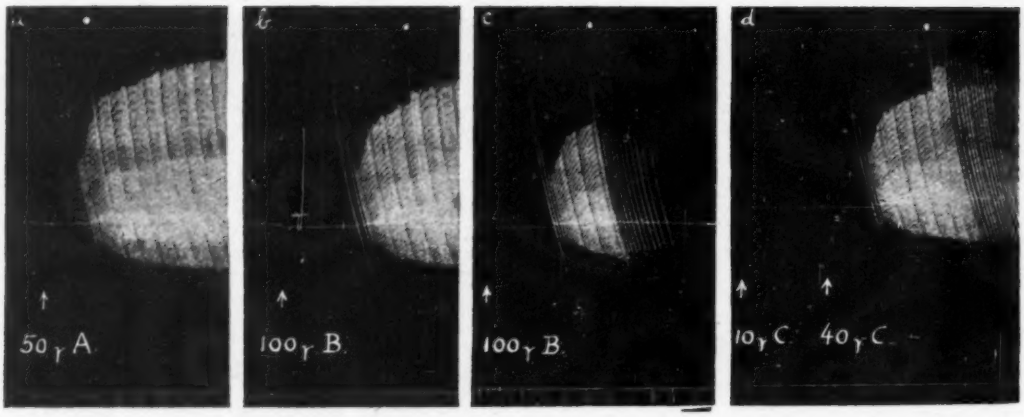

FIG. 2.—Isolated rabbit auricles stopped by paludrine (8 mg.) in 18 min. (*a*) Restarted with acetylcholine (50 μg.) and stopped again with paludrine (8 mg.) in 4 min. (*b*) Restarted with Bovet's acetal compound (100 μg.). Stopped in 4 min. with paludrine (8 mg.). (*c*) Restarted with Bovet's acetal compound (100 μg.). Stopped when washed out. (*d*) Restarted with carbaminoylcholine (50 μg.). Irregular and slow after washing out. Time 30 sec. Dots above record indicate that the bath was changed to fresh Ringer-Locke solution.

initial amplitude (Fig. 1*b*). At this point acetyl-β-methylcholine caused a transient inhibition, followed by a stimulation of the beat. After washing out, acetylcholine also augmented the beat (Fig. 1*c*). There was no change in rate during these stimulations. The bath was replaced by Ringer-Locke solution containing the same concentration of paludrine; the beat of the auricles soon stopped. The arrest was not gradual, but abrupt. Both acetylcholine and acetyl-β-methylcholine restarted the contractions of the auricles (Fig. 1*d*); these stopped when the fluid in the bath was changed to fresh Ringer-Locke solution. Acetyl-β-methylcholine was again used to restart the beat (Fig. 1*f*). It was allowed to act for a longer time than before (Fig. 1*d*), and when the bath was washed out the auricles did not stop beating but the beat became gradually much weaker. Acetyl-β-methylcholine once more stimulated the beat (Fig. 1*g*). It has been repeatedly noticed in this, and in previous work, that if the substance which initiated the beat was removed within 90 sec. of the first contraction, then the beat stopped almost immediately (see Figs. 1*d* and 2*c*). If the substance was allowed to act for a longer time (2–5 min.) then washing out only reduced the beat, and if the drug was left in the bath for more than 5 min. washing out had little effect on the beat.

Adrenaline.—Adrenaline also restarted the beat, as is shown in Fig. 1*e*. The difference between the effect of adrenaline and that of a choline derivative (Fig. 1*f*, acetyl-β-methylcholine) should be noted. Contractions started with adrenaline quickly reached a large amplitude, but then diminished to a small value. When the contractions were started with an acetylcholine-like compound, after the first few beats the amplitude of the contractions steadily increased to a much larger value than that obtained with adrenaline.

Bovet's acetal compound.—Bovet (1944) introduced a new compound, which he claimed had the muscarine-like properties, but no nicotine-like properties of acetylcholine. The formula of Bovet's acetal compound is shown below:

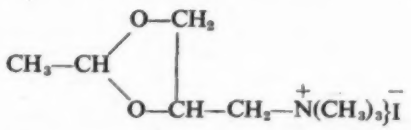

Fig. 2 shows that this, as well as acetylcholine, would restart the auricles which had been stopped with paludrine. The auricles were started with acetylcholine (Fig. 2*a*) but did not stop when washed out. Paludrine was added again, and the beat stopped in 4 min. The bath was washed out, and then Bovet's acetal compound restarted the contractions (Fig. 2*b*). Paludrine again stopped the beat in 4 min.; Bovet's acetal compound again restarted it. This time the bath was washed out within 90 sec. of the initial contraction, and the beat stopped almost immediately.

Carbaminoylcholine.—Fig. 2*d* shows that carbaminoylcholine also restarted the contractions. When the bath was changed, the beat became slow and irregular and did eventually stop.

Choline.—Choline restarted the beat. Fig. 3 shows the record of an experiment in which acetylcholine (100 μg.) started the rhythm (Fig. 3*a*). The bath was washed out twice and the contractions stopped in 4 min. Choline (5 mg.) had no effect, but choline (50 mg.) added 90 sec. later restarted the beat (Fig. 3*b*). When the bath was washed out, the contractions again stopped; they were restarted with acetylcholine (50 μg.).

Benzoylcholine.—Benzoylcholine, which is said to have only nicotine-like properties, failed to restart the contractions of the auricles.

Nicotine.—Nicotine was added (1, 10, and 50 mg.), but this did not restart the beat of the quiescent auricles. After the nicotine had been washed out, two doses of acetylcholine (100 μg.) were needed to restart the contractions, the force of which was much weaker than before the nicotine (Fig. 3*c*). The beat stopped when the bath was changed, but restarted again with acetylcholine (100 μg.) and became more regular on addition of acetylcholine (200 μg.). Nicotine (0.5, 1, and 2 mg.) also failed to restart the contractions of the auricles of which Fig. 2 is the record.

Atropine.—Atropine (500 μg.) added to the bath (Fig. 3*c*) made the beat irregular and the amplitude gradually decreased. The rhythm improved, however, when more acetylcholine was added (1 mg.). The effect of atropine on auricles restarted with acetylcholine depended upon the time at which it was added. If the atropine was added within 90 sec. of the initial contraction, then the beat stopped immediately. This is recorded in Fig. 4. Acetylcholine restarted the beat, which stopped when the bath was changed. The contractions were again started with acetylcholine, but stopped immediately atropine was added. After the bath had been washed out, although acetylcholine did not restart the beat, adrenaline did, and the contractions were unaffected by atropine.

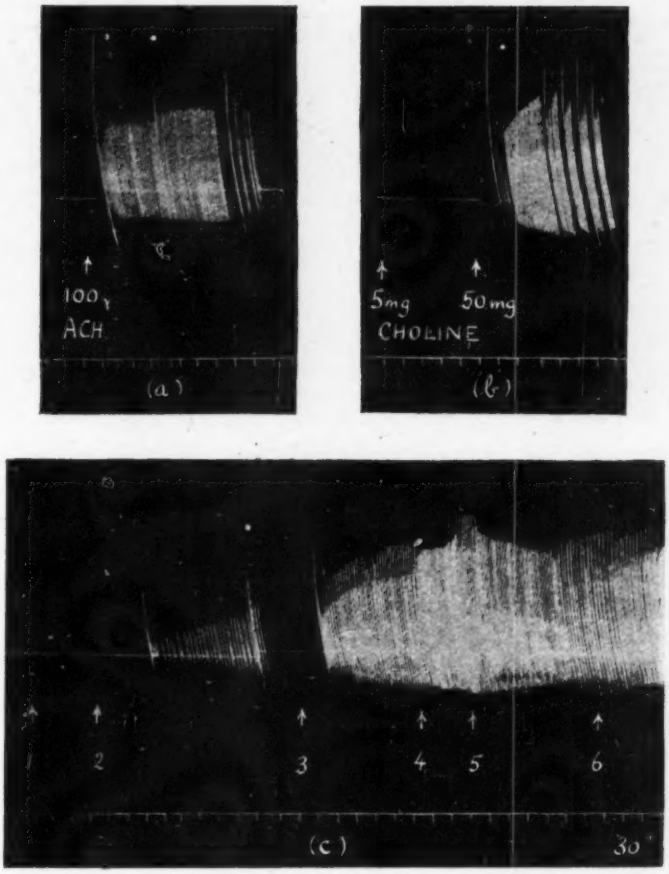

FIG. 3.—Auricles stopped with paludrine (8 mg.) in 26 min. (*a*) Restarted with acetylcholine (100 μg.); stopped on washing out. (*b*) Restarted with choline (55 mg.); stopped on washing out. Between (*b*) and (*c*), restarted with acetylcholine (50 μg.), but failed to restart with nicotine (1, 10, and 50 mg.). Washed out. (*c*) Acetylcholine (100 μg. at 1 and 2), started beat, but not so well as in (*a*). Beat stopped when bath was washed out; restarted with acetylcholine (100 μg. at 3) and improved with acetylcholine (200 μg. at 4). Atropine (500 μg. at 5) made the beat irregular and the amplitude decreased. The beat seemed to improve when more acetylcholine was added (1 mg. at 6). Time 30 sec. Dots above record indicate that bath was changed to fresh Ringer-Locke solution.

Effect of adrenaline on acetylcholine response.—The beat of the auricles was stopped with paludrine and restarted with acetylcholine. After 10 min. the acetylcholine was washed out; the auricles were left for a further 20 min. The amplitude of the beat was then as shown at the beginning of Fig. 5. Adrenaline increased the amplitude and rate of beat to approximately the value previous to treatment with paludrine, and at this point acetylcholine (10 μg.) inhibited both the amplitude and rate of beat. Without any adrenaline in the bath, acetylcholine (10 μg. and 50 μg.) had no effect on the amplitude, whilst acetylcholine (100 μg.) caused a slight increase. This was accompanied by a decrease in rate. The in-

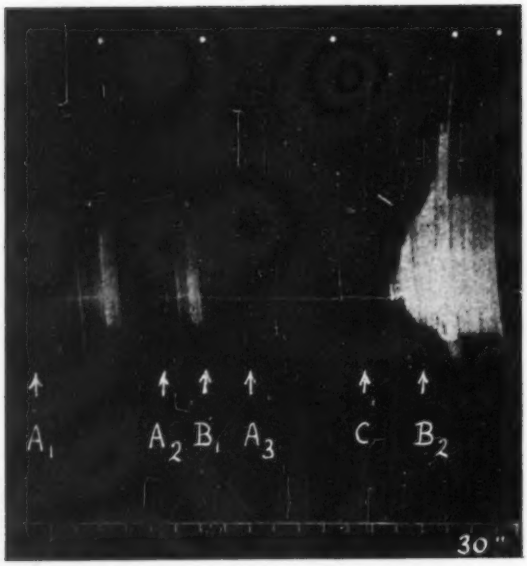

FIG. 4.—Isolated rabbit auricles. The beat was stopped with paludrine (8 mg.) in 27 min. A_1 Started with acetylcholine (50 μg.). Stopped when washed out. A_2 Restarted with acetylcholine (50 μg.). Stopped on the addition of atropine (500 μg. at B_1). A_3 Acetylcholine (50 μg.) failed to restart the beat, but adrenaline (20 μg.) at C started the contractions, which were unaffected by atropine (500 μg. at B_2). Time 30 sec. Dots above record indicate that the bath was changed to fresh Ringer-Locke solution.

hibition with acetylcholine (10 μg.) was repeated in the presence of adrenaline.

DISCUSSION

The possibility that acetylcholine has an integral function in the mechanism of the heart beat has already been discussed in a previous paper (Burn and Vane, 1948). In this paper it was shown that when the beat of isolated rabbit auricles was stopped with paludrine, it could be restarted with acetylcholine. It is interesting to note that Eurico-Paes and Soares (1940) found that chick-embryo heart-cultures which had ceased to beat could be restarted with a mixture of adrenaline and acetylcholine, but not with either drug separately, and that Singh, Sehra, and Singh (1945) published a record of the resumption of contractions of a frog's heart when acetylcholine was added.

It has now been shown that adrenaline and several acetylcholine-like compounds will also restart the beat of auricles stopped with paludrine. That the mechanism of this resumption cannot be due to the release of an adrenaline-like substance (described by Hoffmann *et al.*, 1945; McNamara *et al.*, 1948) is suggested for the following reasons:

(*a*) The release of an adrenaline-like substance is a nicotine-like action of acetylcholine. Nicotine did not restart the beat of the auricles; nor did benzoylcholine, which is said to have only nicotine-like properties. Bovet's acetal compound, however, which he claims has no nicotine-like action, restarted the beat.

(*b*) When the beat was restarted with adrenaline, in most cases the amplitude was initially stimulated, but then declined and remained small (Fig. 1*e*). Contractions restarted with acetylcholine-like compounds, however, steadily increased to a large value (Fig. 1*f*).

As would be expected, atropine prevented the start of the beat with acetylcholine, but not with adrenaline. If atropine was added to the auricles which had just started to contract under the influence of acetylcholine, then the contractions stopped; if the addition of atropine was delayed until about 5 min. after the contractions had started, then it only slowed the beat or had no effect. The same effect could be obtained by washing out; if the bath was washed out within 90 sec. of the first contraction, then the beat stopped; if the bath was not washed out until about 5 min. after the first contraction, then the beat continued.

Thus the mechanism of contraction seems to be both initiated by, and dependent upon, the added acetylcholine for the first few minutes; the beat then becomes independent of the added acetylcholine. This is interesting in view of the work of Abdon and his collaborators, who found that acetylcholine was present in the form of a precursor in cardiac tissue and suggested that there was a constant breakdown to, and reformation from, acetylcholine. They postulated that this "precursor was necessary for the contractions of the cardiac muscle" (Abdon and Hammarskjöld, 1944; Abdon, 1945).

After treatment with paludrine, the normal inhibitory effect of acetylcholine could be restored by first increasing the force and rate of beat with adrenaline. This suggests that the action of acetylcholine may depend upon the rate of metabolism of the myocardium, or the actions of adrenaline and acetylcholine on the heart are interdependent after treatment with paludrine.

SUMMARY

1. Various drugs have been tested to find whether they will restart the contractions of isolated rabbit auricles, previously stopped with paludrine.

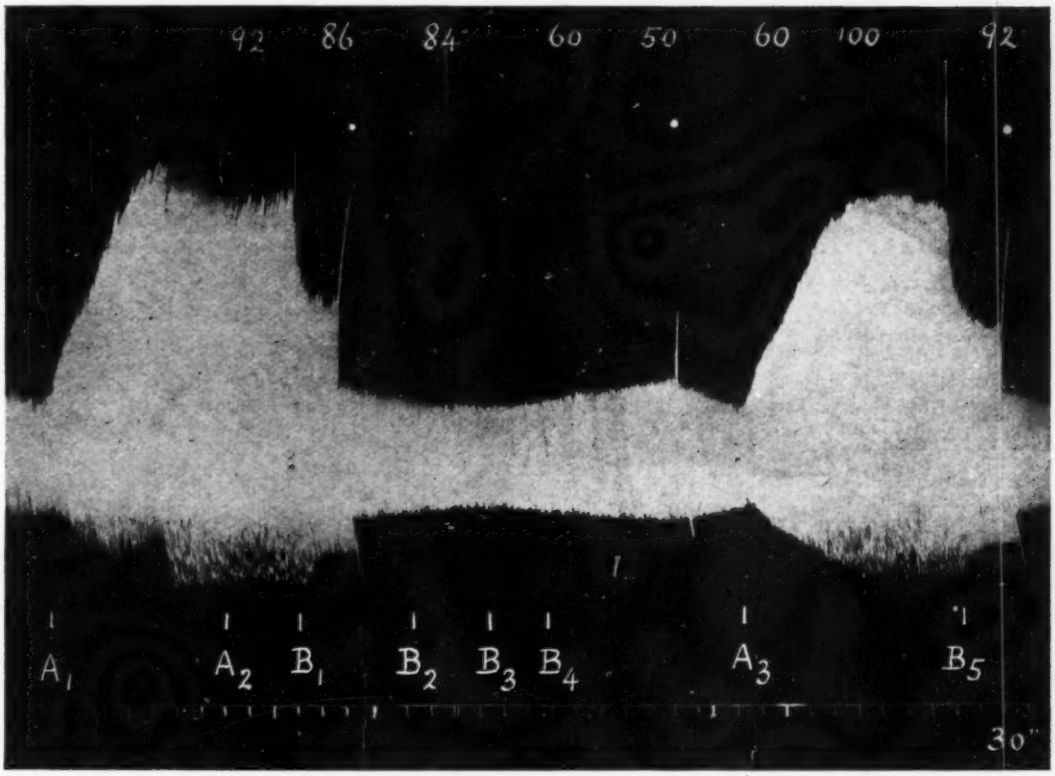

FIG. 5.—After exposure to paludrine the beat of the auricles stopped, but was restarted with acetylcholine (100 μg.). This was washed out after 10 min. Auricles were left for 20 min. At A_1, adrenaline (20 μg.) increased the rate and amplitude. Further adrenaline (20 μg. at A_2) had no effect, but acetylcholine (10 μg. at B_1) caused inhibition of beat. After washing out, the amplitude returned to its previous value, and at this stage acetylcholine (10 μg. at B_2 and 50 μg. at B_3) gave no inhibition of amplitude, although the rate was decreased. Acetylcholine (100 μg. at B_4) caused slight augmentation of amplitude. After washing out, adrenaline (20 μg. at A_3) again increased the rate and amplitude; acetylcholine (10 μg. at B_5) again inhibited the beat. Figures along top indicate rate of beat per min. Time 30 sec. Dots above record indicate that the bath was changed to fresh Ringer-Locke solution.

2. Of these drugs, choline, carbaminoylcholine, acetyl-β-methylcholine, Bovet's acetal compound, and adrenaline all restarted the beat.

3. The beat was not started by nicotine or benzoylcholine.

4. After treatment with paludrine the normal inhibitory effect of acetylcholine could be restored by first adding adrenaline to the auricles.

5. The results are discussed.

I am deeply indebted to Professor J. H. Burn for his help and guidance throughout this work, which was carried out during the tenure of a grant from the Therapeutic Research Corporation of Great Britain. The paludrine was kindly supplied by Dr. F. L. Rose of Imperial Chemical (Pharmaceuticals) Limited.

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Brit. J. Pharmacol. (1948), **3**, 346.

THE INHIBITORY ACTION OF PALUDRINE ON THE SECRETION OF GASTRIC JUICE

BY

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(Received July 22, 1948)

Because excessive secretion of gastric juice is a common concomitant of chronic peptic ulcers, various workers have looked for substances having an inhibitory action. The secretion of HCl, though partly controlled by the vagus nerves, is peculiar in being stimulated by histamine and in being not greatly affected by atropine. The effect of atropine is described differently by different observers (Polland, 1930 ; Atkinson and Ivy, 1937 ; Gray, 1937 ; King, Comfort, and Osterberg, 1944). It appears to be more effective in abolishing the secretion produced by giving a meal than that produced by injecting histamine. Davenport (1940), and later Rehm and Enelow (1945), investigated the effect of sodium thiocyanate ; when given intravenously to dogs in sufficient amount, it was found to inhibit the secretion of HCl completely.

Recently Babkin and Karp (1947) have observed that the two antimalarial substances quinine and mepacrine (atebrin), when injected intravenously into dogs, depressed the secretion of gastric juice produced by stimulation of the vagus nerves. The doses they used were large in relation to the doses ordinarily given to man ; thus a dog of 10 kg. received 0.2 g. quinine bisulphate or 75 mg. mepacrine. When secretion was produced by the injection of histamine, neither quinine nor mepacrine affected it.

Recently a simple method of examining substances for an inhibitory action on gastric secretion has been developed in this laboratory by Wood (1948) ; cats are used, anaesthetized by cyclopropane or pentobarbitone (nembutal). The only essential point in which the method differs from that proposed by Lim (1923), and modified by Roth and Ivy (1944), is that histamine solution has been infused into the jugular vein at a uniform rate for periods up to 7 hours. In order to obtain a uniform infusion, a pump designed and made by Dr. E. H. J. Schuster has been used, with which a reasonably steady secretion of gastric juice has been obtained. The oesophagus was tied in the neck and not at the cardiac orifice, to avoid the vagi.

The action of paludrine

During the examination of the pharmacological properties of paludrine, its effect on the secretion of gastric juice was tested by this method. When histamine was infused, the rate of secretion rose gradually until at the end of about 1½ hours the amount collected in each 10 min. period became fairly steady. Sometimes 4 hours passed before the flow was steady. At this point, while the histamine infusion continued, the effect of paludrine

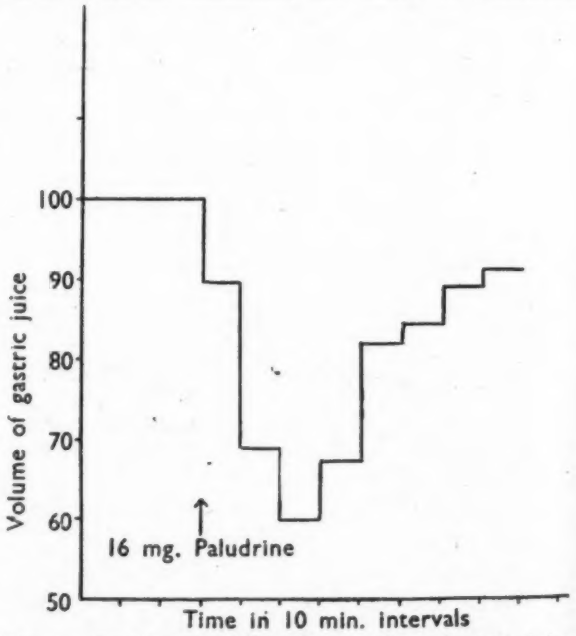


FIG. 1.—To show the fall in the secretion of gastric juice when 16 mg. paludrine were injected. The mean effect on the volume of juice in 9 cats is shown. In each experiment the initial secretion has been given the value 100. Abscissae: time in 10 min. periods.

was examined by making an injection of a single dose intravenously, or else by infusing paludrine intravenously. It was found that paludrine in doses which had only a transient effect on the blood pressure diminished the volume of juice secreted for a period of 60–90 min. The results of nine experiments in which a single injection of 16 mg. paludrine was given were combined so that the mean effect could be determined. This effect is shown graphically in Fig. 1 ; the volume of juice excreted per 10 min. during 30 min. before paludrine was injected is expressed as 100, and the volume fell to 60, after the injection. After reaching a minimum the flow gradually returned to its previous value, and the effect of paludrine could then be observed a second time. Details of one experiment were as follows: Histamine was infused at the rate of 15 μg. per min. into a cat of 3.2 kg. under nembutal anaesthesia (25 mg. per kg.). The rate of flow of gastric juice was 2.6 ml. per 10 min. during 30 min., and after the injection of 16 mg. paludrine it fell to 1.6 ml. during 65 min. The rate then returned to 2.6 ml. for a further 60 min.

In four experiments the injection of 8 mg. paludrine caused a smaller mean reduction in the flow to 75 per cent of the initial value.

When larger amounts of paludrine were given by slow infusion instead of by single injection, the periods of reduced gastric secretion lasted until the termination of the experiment. Fig. 2 shows the mean effect of infusing 60 mg. paludrine during 30 min. into 6 cats. The graph records the change in the total free acid excreted. The volume of juice per 10 min. was measured ; 1 ml. of this juice was then titrated against $N/50$ NaOH using thymol blue as the indicator. The total secretion of free acid per 10 min. was then calculated. The initial rise in secretion shown in Fig. 2 occurred in two of the six experiments for 20 min. ; thereafter there was a prolonged fall. In these experiments, both the volume of juice secreted and the concentration of free acid in the juice were reduced. The only effect the paludrine infusion had on the blood pressure was to cause an occasional initial rise. In four other experiments, doses of paludrine from 120 mg. to 180 mg. were infused. Table I shows these results. It can be seen that there was an initial stimulation of the secretion in Exp. 1. In these experiments, as in those recorded in Fig. 2, the volume of juice secreted did not recover from its low value.

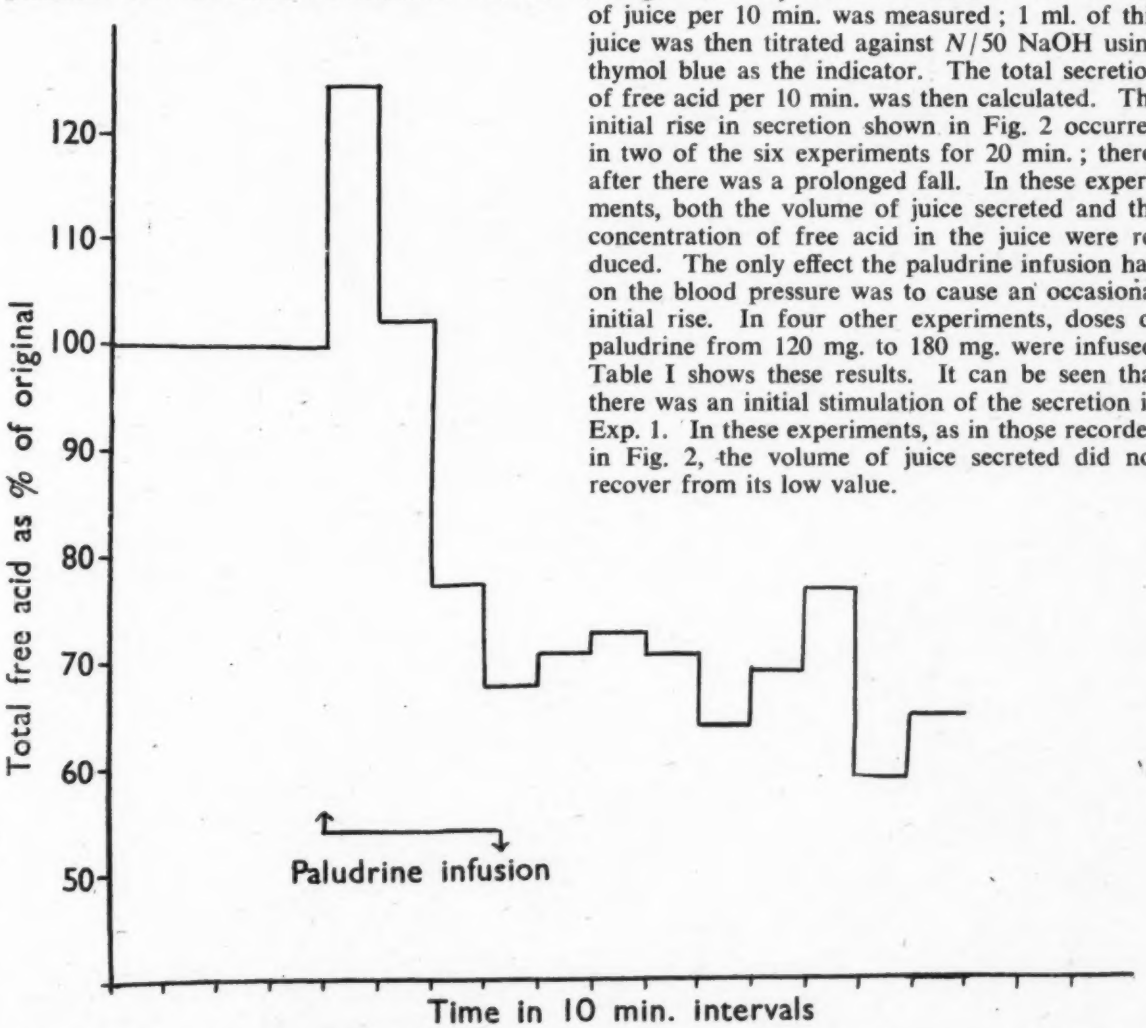


FIG. 2.—To show the mean fall in total free acid in gastric juice caused by intravenous infusion of 60 mg. paludrine into 6 cats. In 2 experiments there was an initial increase in secretion.

TABLE I

EFFECT OF INFUSED PALUDRINE ON VOLUME OF GASTRIC JUICE IN CATS

Exp.	Rate of histamine infusion μg./min.	Paludrine mg.	Duration of infusion min.	Gastric juice per 10 min. ml.	Period of observation min.	Mean percentage to which volume of juice fell
1	15	140	52	6.3 8.7	60 20	138
		"	"	4.3	110	68
2	15	120	100	4.6 3.8	30 140	82
3	15	180	90	4.7 2.2	60 160	47
4	15	120	60	1.7 0.5	40 170	29

In all experiments the rate of histamine infusion was usually 15 μg. per min., or more, throughout. Only in two experiments was it 10 μg. per min. This is a high rate of histamine infusion, providing a powerful stimulus to secretion and a severe test for an inhibitory substance.

Paludrine consists of the biguanide molecule, substituted at one end with the *p*-chlorophenyl radical, and at the other with the *iso*propyl radical. Compounds structurally related to paludrine were also tested to see if they would inhibit gastric secretion. The first three compounds tested were biguanide, *iso*propyl biguanide, and di-*iso*propyl biguanide. All were found to be inactive as shown by the results in Table II. These results were obtained by expressing the total secretion for an hour after the drug was given as a percentage of the total secretion of the hour previous to the administration.

TABLE II

NON-INHIBITORY BIGUANIDES

Substance	Dose in mg.	Total free acid for 1 hr. after drug as % of that for 1 hr. before drug	Average
Biguanide ..	60 60 60	133 91 84	106
*Iso*propyl-biguanide	60 60 60 60	140 106 103 57	102
	120	89	
Di-*iso*propyl-biguanide	60 60	120 93	107

In an effort to obtain a more quantitative estimation of the inhibitory power, the principle of the method recently described by Howat and Schofield (1948) was adopted. The main difference between this method and that previously used was that, instead of giving the test substance during a constant infusion of histamine, it was given between two periods of histamine infusion, each of 48 min. duration. The second response was expressed as a percentage of the first, the

TABLE III

PALUDRINE AND RELATED COMPOUNDS

Substance	Dose infused mg.	Total free acid expressed as % of original value	Average
Paludrine .. .. (N_1-*p*-chlorophenyl-N_5-*iso*propyl-biguanide)	60 60 60	75 71 17	54
N_1-*p*-chlorophenyl-N_5-methyl-biguanide	60 60 32	66 32 68	49
N_1-*p*-chlorophenyl-biguanide	80 60 60	92 33 155	93
N_1-*p*-methoxyphenyl-biguanide	60 60	78 64	71
N_1-*p*-chlorophenyl-N_5-methyl-guanidine	60 60 60	141 70 23	78

TABLE IV

SUMMARY OF RESULTS

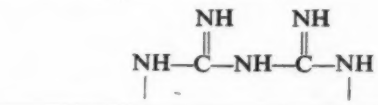
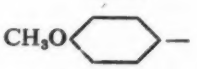
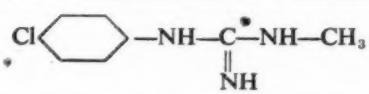
	Amount (mg.) administered	No. of expts.	Approximate percentage to which secretion was reduced
—C_3H_7	60	9	60
—CH_3	60	2	50
 —H	60	2	70
—H	60	3	90
C_3H_7— —H	60	4	no reduction
C_3H_7— —C_3H_7	60	2	" "
H— —H	60	3	" "
	60	3	80

figure being a measure of the inhibitory power of the test substance.

By this method, five more compounds were tested (including paludrine as a standard). The results of these experiments are shown in Table III. The mean secretion of acid in three experiments in which paludrine was infused was reduced to 54 per cent of the original value; by the first method, six experiments gave a mean value of 64 per cent. The results shown in Tables II and III have been combined and arranged in order of activity, to give Table IV.

Discussion and Summary

Extensive trials of paludrine in men have been made in testing its value against malaria, and it is known to be a well-tolerated substance. A large dose produces gastro-intestinal symptoms in some individuals, and Hughes, Schmidt, and Smith (1947) have described how dogs fed on a diet containing paludrine become disinclined to eat. It seems probable that these observations are related to our finding that paludrine exerts an inhibitory action on the volume and acidity of gastric juice evoked by histamine. A similar compound in which the *iso*propyl group of paludrine was replaced by a methyl group was found to exert a similar effect. Other related substances had less or no effect. We have observed an initial stimulating action in a few experiments, preceding the inhibition. This is probably an example of a preliminary stimulant action preceding inhibition, which is commonly met in inhibitory drugs. Thus the first effect of taking atropine by mouth is to slow the pulse rate and cause the hands to become moist in most people; the later effect is of course the opposite. Atropine appears first to stimulate and later to paralyse the cholinergic nerve endings in the heart and sweat glands.

We are greatly indebted to Dr. F. L. Rose, of Imperial Chemical (Pharmaceuticals) Limited, for the substances which we have examined. The work was done while one of us (J. R. V.) was in receipt of a grant from the Therapeutic Research Corporation.

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Brit. J. Pharmacol. (1948), 3, 350.

THE EFFECT OF PALUDRINE ON GASTRIC SECRETION IN MAN

BY

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(Received July 22, 1948)

In order to investigate the effect of paludrine on human gastric secretion the following experiments were carried out on 9 students. Each subject, who had not taken food for at least four hours, had his stomach emptied by aspiration. He was then given a meal of 450 ml. gruel, and samples of gastric contents were withdrawn every 15 minutes for 2 hours. The hydrochloric acid content of each sample was measured by titration against standard sodium hydroxide using thymol blue as indicator. Three control experiments were first carried out on each subject in order to obtain his normal response.

Paludrine was given by mouth, either as tablets or in cachets, 2 or 4 hours before the test meal. The only toxic effect was slight nausea in four subjects after the higher doses, and this passed off before the meal was given. Results were assessed as follows (see Table I). The amount of HCl in each sample of gastric juice, expressed as ml. $N/10$ HCl per 100 ml. juice, is set out in each column, and the mean of these figures is shown at the bottom of the column. Mean figures of the three control experiments are given in column (v). The results after paludrine are given in columns (vi) and (vii) and can be compared with those of the controls.

Table II shows the results obtained on each student in this way. It can be seen that when paludrine was given 4 hours beforehand (columns (vi) and (vii)) there was usually no effect. In one case, student J, the acidity was markedly increased. When, however, 0.9–1.0 g. paludrine was given 2 hours before the test meal (columns (viii) and (ix)) there was a decrease in the gastric acidity of every subject but one. The mean figure for the acidity of the samples when this dose of paludrine was given two hours before the test meal was 23.0 ml. per 100 ml. juice. This is 60 per cent of the mean figure of the control samples (38.1 ml.).

TABLE I

STUDENT H

The figures in columns (ii)–(vii) are ml. $N/10$ HCl per 100 ml. gastric juice

(i)	(ii)	(iii)	(iv)	(v)	(vi)	(vii)
Min.	Controls			Mean of controls	Paludrine	
	1	2	3		0.9 g. 4 hr. before	1.0 g. 2 hr. before
15	0	0	8	2.8	8	8
30	23	15	40	26.0	28	22
45	43	58	40	47.0	50	48
60	67	80	80	76.0	83	65
75	80	78	50	69.3	100	53
90	73	73	58	68.2	80	42
105	69	32	85	62.0	53	25
120	43	—	57	50.0	65	37
Mean	49.7	48.0	52.2	50.1	58.4	37.5

TABLE II

EFFECT OF PALUDRINE ON GASTRIC SECRETION OF HCl

The figures are ml. $N/10$ HCl per 100 ml. gastric juice

(i)	(ii)	(iii)	(iv)	(v)	(vi)	(vii)	(viii)	(ix)
Subject	Controls			Mean of controls	Dose of paludrine Time before meal			
	1	2	3		0.3 g. 4 hr.	0.9 g. 4 hr.	0.9g. 2 hr.	1.0g. 2 hr.
A	20	35	10	22	28	26		8
B	45	45	30	41	37	45		26
C	28	39	26	32	43		13	27
D	52	33	33	39		29		25
E	46	71	73	64	37	82		47
F	18	41	46	37	36	13		17
G	28	49	17	33			14	11
H	49	48	52	50		58	37	
J	25	26	24	25	51	54		28
Mean	34.5	43.0	34.5	38.1	38.6	43.8		23.0

An analysis of variance of the figures in Table II shows that this reduction of acidity was highly significant ($P = < 0.01$) by the variance-ratio test.

Summary

1. The effect of paludrine on gastric acidity has been investigated by carrying out test meals on 9 subjects.

2. A significant reduction in acidity occurred when 0.9–1.0 g. paludrine was given by mouth 2 hours before the test meal.

We are grateful to the nine students who volunteered to act as subjects in these experiments.

This work was done when one of us (J. R. V.) was receiving a grant from the Therapeutic Research Corporation of Great Britain; and when another of us (J. M. W.) was receiving a personal grant from the Medical Research Council.

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THE EFFECT OF PALUDRINE ON HUMAN GASTRIC SECRETION

BY

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(Received July 22, 1948)

Burn and Vane (1948) have reported that paludrine depressed gastric secretion in cats after histamine stimulation. This was worth investigating in man, as paludrine is comparatively non-toxic and a means of reducing gastric secretion might be of value in the treatment of peptic ulcer.

Doses of up to 1.5 g. a day have been given without producing serious toxic effects. Abdominal discomfort, nausea, and vomiting have been noted by Fairley (1946) and Maegraith (1946) with doses of 1.0 g. a day, and Fairley also observed diarrhoea and haematuria. The toxic symptoms usually subsided without reduction of the dose as the malaria for which the paludrine was given was relieved.

We have studied the effect of paludrine on gastric secretion in man by two methods, giving it orally before a gruel test meal and intravenously with subcutaneous injections of histamine.

Twenty patients (11 men and 9 women) were given gruel test meals on two consecutive days, on one of which they received 1.0 g. paludrine hydrochloride by mouth 2 hours before the meal began. One man and one woman were excluded from the series on account of achlorhydria in both test meals, and the tests could not be completed in two women who vomited after the paludrine. The results in 16 cases are available for analysis.

Paludrine was given before the first meal in half the cases and before the second in the other half. The resting juice was drawn off immediately before the meal was started, and samples were taken at half-hourly intervals up to two and a half hours. The concentration of free acid after paludrine was compared with that of the sample drawn off after the same interval in the control meal. The averaged results for the 16 cases are shown in Table I.

TABLE I

The effect of 1.0 g. paludrine hydrochloride on the concentration of free HCl after a gruel test meal. Mean results for 16 patients.

Time	C.C. *N*/10 HCl per 100 c.c. gastric juice		B—A	"*t*"
	A Control	B Paludrine		
½ hr.	18.1	7.7	−10.4	2.01
1 hr.	30.8	17.3	−13.5	2.17
1½ hrs.	37.7	25.4	−12.3	2.31
2 hrs.	30.6	25.5	−5.1	1.12
2½ hrs.	22.3	23.7	+1.4	—
Average	27.9	19.9	−8.0	3.78

Individual results varied from an increased concentration of acid after paludrine to the production, in four cases, of achlorhydria. Considering them as a whole, the average depression of free acidity after paludrine was 8.0 c.c. *N*/10 HCl per 100 c.c. juice. Applying the "*t*" test to the 80 individual differences between comparable specimens, the probability of obtaining such differences due to chance on the hypothesis that paludrine had no effect is less than 0.01 ($t=3.78$, $n=79$). The depression can therefore be considered to be real. If the results at each period after the start of the meal are considered separately a significant depression with paludrine was found at 1 and 1½ hours ($0.02 < P < 0.05$), while after a half-hour the depression was just too small to be considered significant. No effect was demonstrated at 2 and 2½ hours.

Repetition of the test meal had no effect on the acidity of the gastric secretion. The average acidity of all the samples drawn off during the second meal was 0.3 c.c. *N*/10 HCl per 100 c.c.

* While working with a grant from the Medical Research Council.

less than the average of the samples of the first meal.

In contrast to the results with oral paludrine and gruel test meals, no effect was demonstrated when paludrine was given intravenously and gastric secretion was stimulated with histamine subcutaneously; 0.5 mg. histamine was given initially, with a further 0.5 mg. after 60 to 80 minutes. Doses of up to 400 mg. paludrine acetate were given intravenously immediately before the second injection of histamine. The stomach was emptied at 10 min. intervals, and the effect of the paludrine was determined by comparing the amounts and average concentrations of free acid secreted in the hours following the first and second injections of histamine. The results in the 4 subjects given 400 mg. are shown in Table II.

TABLE II

The amount and concentration of free acid secreted in one hour after 0.5 mg. histamine (1st hr.) and 0.5 mg. histamine S.C. and 400 mg. paludrine I.V. (2nd hr.).

Subject	Amount of free acid, c.c. *N*/10 HCl			Average concentration of free acid, c.c. *N*/10 HCl % (v/v)		
	1st hr.	2nd hr.	Difference	1st hr.	2nd hr.	Difference
1	202.1	155.4	−46.7	59.0	59.1	+0.1
2	203.0	181.9	−21.1	67.9	65.7	−2.2
3	14.1	19.9	+5.8	20.0	37.0	+17.0
4	254.7	323.3	+68.6	107.0	113.2	+6.2

The amount of paludrine given intravenously was small in comparison with that used by Burn and Vane in cats, but it was comparable with the oral dose which was effective in depressing acid secretion in response to a gruel test meal in man. The reason for the conflicting results is not clear.

Summary

1.0 g. paludrine hydrochloride given 2 hours before the start of a gruel test meal produced a significant depression of more than 33 per cent in the concentration of free acid during the first 1½ hours of the meal. No consistent effect was observed with doses of up to 400 mg. paludrine acetate given intravenously on the gastric secretion in response to histamine.

We should like to express our thanks to Dr. F. Avery Jones, under whose direction the work was carried out, for his help and advice.

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THE ACTION OF ANTAGONISTS OF ACETYLCHOLINE ON THE VESSELS OF THE RABBIT'S EAR

BY

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Our interest in the work to be described began with an observation made in this laboratory by H. W. Ling, that when the vessels of a dog's hind leg were perfused with defibrinated blood containing adrenaline, the injection into the cannula of atropine sulphate caused vasodilatation and increased blood flow through the leg. The observation led to an investigation of the vascular action of atropine by Bussell (1940), who found in the course of his observations that the constrictor action of adrenaline on the vessels of the rabbit's ear, perfused with Locke's solution, was abolished by atropine, though atropine did not affect the constrictor action of posterior lobe extract.

Since that work was done, fresh light has been thrown on the action of atropine by the work of Dawes (1946), who showed that it exerted a quinidine-like action on the rabbit auricles, and that its properties were shared in varying degrees by other substances, including local anaesthetics such as procaine, and the analgesic pethidine. These substances revealed themselves as antagonists of acetylcholine, and a comparison of the properties of some of them was made by de Elío (1948); a study of their effect on body temperature was also made by Dutta (1948). It remained to investigate their vascular action and to observe in particular whether like atropine they would abolish the constrictor action of adrenaline in the vessels of the rabbit's ear. The substances used have been procaine, pethidine (demerol), benadryl, quinidine, and atropine, though a few observations have also been made with antistin, anthisan (neoantergan), and *iso*conessine. Since Gowdey (1948) had observed that priscol (benzylimidazoline) reversed the action of adrenaline in the rabbit's ear, we studied the effect of this substance also.

METHODS

All observations were made on the vessels of the rabbit's ear perfused with Locke's solution. The method of Gaddum and Kwiatkowski (1938) was used first in order to stimulate the postganglionic sympathetic fibres. Later experiments were carried out by perfusing the ear severed from the head according to the method of Rischbieter (1913) (originally introduced by Bissemski). The outflow recorder described by Stephenson (1948) was found well suited to record small changes in the diameter of the vascular bed, which would not have been observed using the drop-timer.

RESULTS

The abolition of the vasoconstrictor action of adrenaline

Injections of atropine sulphate, benadryl, pethidine hydrochloride (demerol), procaine hydrochloride, and quinidine hydrochloride were found to diminish or abolish not only the vasoconstriction produced by the subsequent injection of adrenaline but also that produced by sympathetic nerve stimulation. These effects are illustrated in Figs. 1 and 2. In Fig. 1 the effect of 200 μg. pethidine was transitory, being present 2 min. after its injection but absent 8 min. later. In Fig. 2 the effect of 500 μg. quinidine was still present, though fading, 28 min. after its injection. C. S. Jang (1940) had previously observed that procaine antagonizes adrenaline and sympathetic nerve stimulation on blood vessels. The amounts of the different substances required to reduce the effect of sympathetic nerve stimulation were in general the same as those required to reduce the effect of adrenaline, but this was not true for atropine. Whereas 5 μg. atropine abolished the effect of

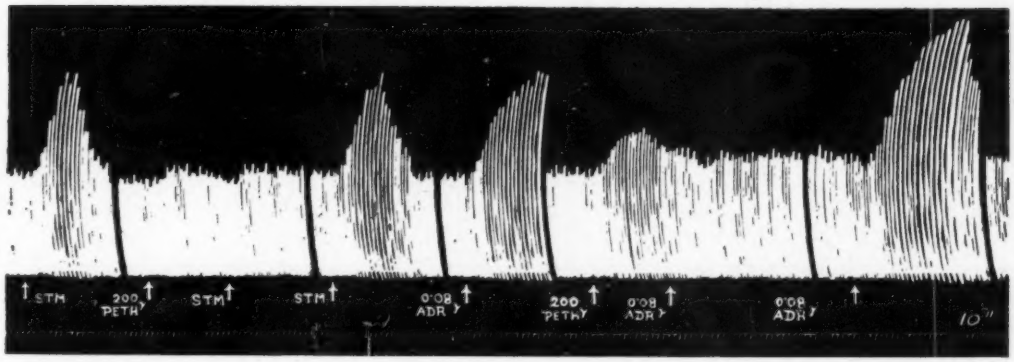

FIG. 1.—Rabbit ear perfusion using Gaddum's drop-timer. Vasoconstriction produced by sympathetic stimulation; after injection of 200 μg. pethidine, one stimulation was without effect; the next stimulation was again effective. Vasoconstriction produced by 0.08 μg. adrenaline; 200 μg. pethidine when injected caused some vasoconstriction; the next injection of 0.08 μg. adrenaline was without effect, but the succeeding injection produced augmented vasoconstriction.

TABLE I

Adrenaline hydrochloride	Histamine acid phosphate	Vasoconstriction abolished by
0.015 μg.	0.2 μg.	5 μg. pethidine hydrochloride
0.1 μg.	0.5 μg.	10 μg. „ „
0.01 μg.	0.01 μg.	0.5 mg. procaine hydrochloride
0.075 μg.	0.2 μg.	0.2 mg. „ „
0.015 μg.	0.2 μg.	4 μg. benadryl
		0.02 μg. „
		30 μg. quinidine hydrochloride
		0.3 mg. „ „
		4 μg. atropine sulphate
		6 μg. „ „

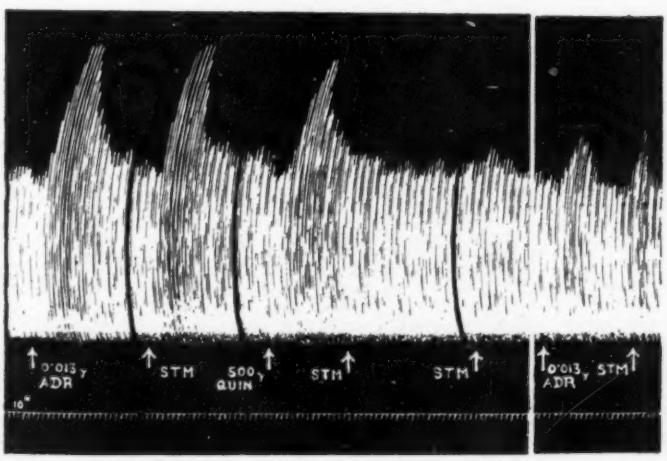

FIG. 2.—Record as Fig. 1. Vasoconstriction produced by 0.013 μg. adrenaline and also sympathetic stimulation. Injection of 500 μg. quinidine caused vasoconstriction, and then stimulation was twice ineffective. Later 0.013 μg. adrenaline caused much less vasoconstriction than before, and stimulation a similar reduced effect.

adrenaline, 2 mg. was required to abolish that of sympathetic nerve stimulation.

Observations were also made on the vasoconstriction produced by histamine; this was also abolished by these substances. The figures given in Table I illustrate the order of the amounts required to abolish the effects of adrenaline and of histamine. Against adrenaline, atropine, benadryl, and pethidine were strongest, quinidine next, and procaine weakest. Against histamine, benadryl was strongest, then atropine and pethidine, and finally procaine and quinidine weakest. Anthisan (neoantergan) and antistin were also observed to abolish the constrictor action of adrenaline.

The abolition of the vasodilator action of adrenaline

The reversal of the constrictor action of adrenaline to a dilator action by perfusing priscol in a concentration of 0.2 mg./ml. (Gowdey, 1948) made it possible for us to examine the effect of these compounds on the dilator action of adrenaline. For these experiments we used Stephenson's recorder. We observed that atropine, benadryl, and pethidine abolished the vasodilator action; the effect of pethidine is illustrated in Fig. 3. We were not able to observe a similar action of procaine and of quinidine because when injected in the presence of priscol they themselves caused vasodilatation, which made it difficult to determine the effect of adrenaline. When, however, a succession of small doses of quinidine was injected, it was possible to see

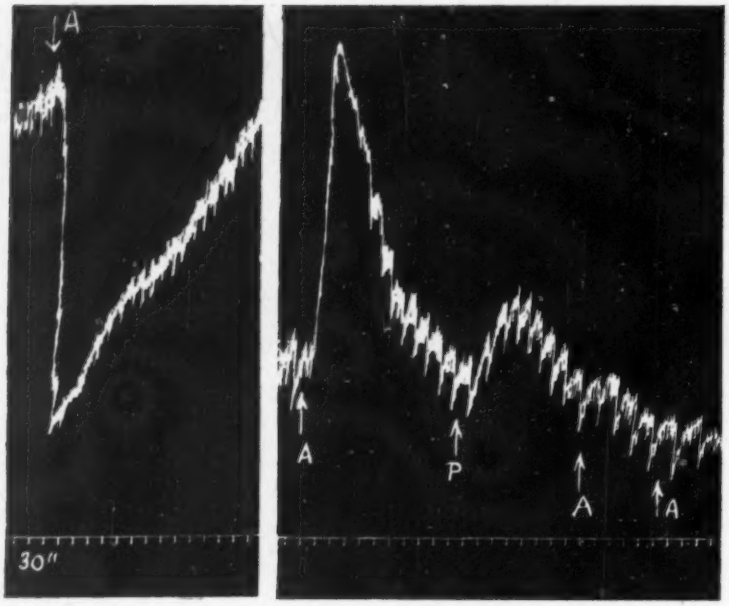

FIG. 3.—Outflow record from ear using Stephenson's recorder. Vasoconstriction due to 0.004 μg. adrenaline (A). Between the two parts of the record priscol was added to perfusion fluid (200 μg./ml.), and adrenaline (0.008 μg.) at A then caused dilatation. At P, 10 μg. pethidine hydrochloride was injected, and 0.008 μg. adrenaline was then twice injected at A; it had no effect.

that the vasodilator action of adrenaline was reduced.

Substances causing adrenaline reversal

We found evidence in the literature that some of the compounds we were considering reverse the constrictor action of adrenaline. Thus Wehland (1924) found that, in the perfused vessels of the frog, atropine reversed the action of adrenaline. Further the observation of Murakami (1930) and Akamatsu (1933) that quinidine reversed the action of adrenaline on the blood pressure of the cat was supported by the evidence of Keogh and Shaw (1943), who found that quinine reversed the action of adrenaline but that reversal by quinidine was more difficult to produce. We therefore carried out experiments on the ear vessels perfused by Locke's solution containing quinidine hydrochloride (20 μg. per ml.) and found that the constrictor action of adrenaline was reversed, as shown in Fig. 4. A similar reversal was also observed when the perfusion fluid contained benadryl (10 μg. per ml.) or procaine hydrochloride (200 μg. per ml.). The effects of pethidine hydrochloride and of atropine sulphate were less clear, for when perfused in a concentration of 10 μg. per ml. and 30 μg. per ml. respectively, the constrictor phase was not wholly abolished, and the vasodilatation which followed was not so well marked as with the other substances. The vasodilatation was nevertheless present, as shown in Fig. 5. In one experiment we confirmed Rothlin's observation (1925) that ergotamine reversed the action of adrenaline, since we observed a reversal with ergotoxine.

Vascular action of benadryl, pethidine, etc.

In the course of these experiments we observed that the injection of benadryl or of the other substances had an effect on the vessels. In Fig. 1 the second injection of pethidine had a small vasoconstrictor effect and in Fig. 2 the injection of quinidine caused vasoconstriction. All except atropine were found to cause constriction, and we observed in addition that the constriction was reversed to dilatation when priscol was present in the perfusing fluid. During perfusion with priscol, atropine also caused dilatation. These effects are illustrated in Figs. 6 and 7. (In one experiment in a freshly prepared ear, benadryl caused slight vasodilatation in the absence of priscol. It seems to us probable that a dilator effect can be observed with these substances at an early stage, but that

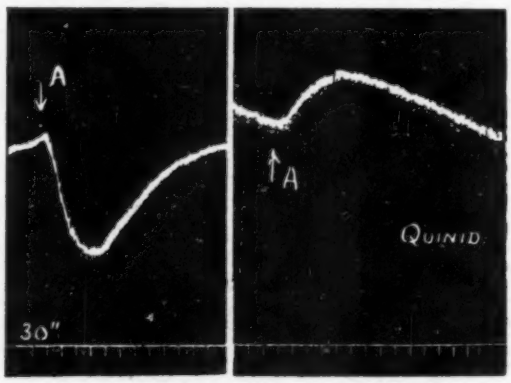

FIG. 4.—In the first part of the record, at A, 0.01 μg. adrenaline caused vasoconstriction. Between the two parts of the record, quinidine hydrochloride was added to the perfusion fluid (20 μg./ml.) and in the second part 0.05 μg. adrenaline at A caused vasodilatation.

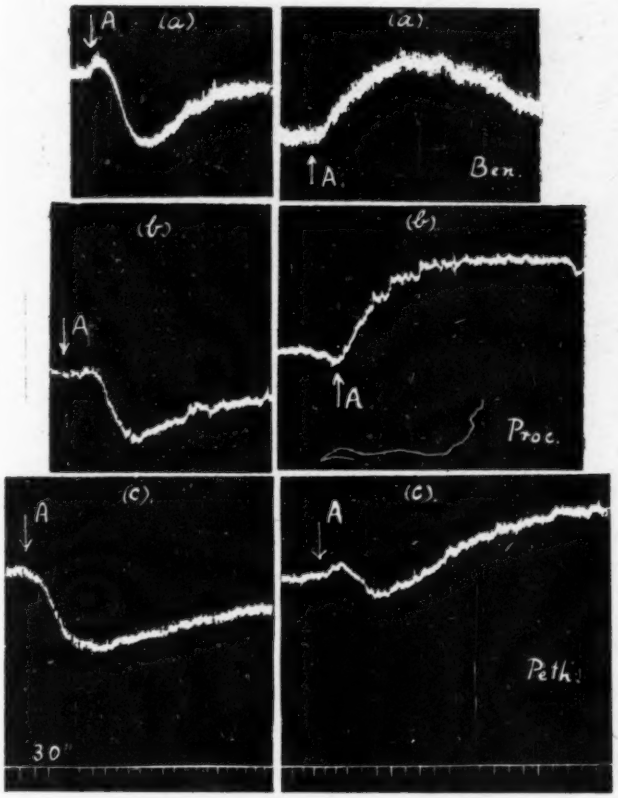

FIG. 5.—Similar to Fig. 4. Reversal of adrenaline (A). Action due to: (a) benadryl perfused in concentration 10 μg./ml., (b) procaine 200 μg./ml., and (c) pethidine 10 μg./ml. In (a) 0.005 μg. adrenaline was injected before and also during benadryl infusion. In (b) 0.01 μg. adrenaline before procaine, 0.02 μg. adrenaline during procaine infusion. In (c) 0.005 μg. adrenaline before pethidine, 0.01 μg. adrenaline during pethidine infusion.

this gives place as perfusion continues to a constrictor effect, as with acetylcholine. This requires further investigation.)

Action of acetylcholine and histamine

When the vessels of the rabbit ear are freshly prepared it is usual to observe that acetylcholine causes vasodilatation during the first 8 hours. At the end of 24 hours, this dilator response usually changes to a constrictor response. We observed that this constrictor action of acetylcholine was also reversed to a dilator action when the perfusing Locke's solution contained priscol (0.2 mg./ml.). This is shown in Fig. 8. On the other hand, the constrictor action of histamine was not reversed, though it was reduced by priscol; thus, before adding priscol to Locke's solution for the perfusion, constriction was produced by 0.03 μg. histamine acid phosphate; during priscol perfusion constriction was produced by amounts from 0.25–1.0 μg.

Exceptional actions of adrenaline and histamine

When adrenaline and histamine are injected constriction is the ordinary response in the freshly prepared organ. We have, however, observed a dilator response to adrenaline and to histamine exceptionally. In one rabbit ear the vessels were in high tone when the perfusion began, and it was necessary to raise the Marriotte bottle to a height of 75 cm. Gradually the perfusion became faster, but the resistance remained considerable and for several hours dilator responses to adrenaline and histamine were consistently recorded, as well as to acetylcholine. These responses are illustrated in Fig. 9. Repeated control observations were made with Locke's solution. On the next morning, however, the dilator responses had disappeared, and all three substances caused vasoconstriction.

In another ear, when freshly prepared, it was observed that adrenaline caused constriction followed by dilatation of rather greater extent than the constriction, and did so after several injections. Here again histamine also caused dilatation, though the effect was very small.

DISCUSSION

Adrenaline is commonly described as a vasoconstrictor substance, histamine and acetylcholine as vasodilator substances. Yet so long ago as 1918 Dale and Richards showed that, when injected into the vein of a cat anaesthetized with ether, all three substances caused the expansion of the volume of a fully denervated hind leg, the expansion being accompanied by a fall in blood pressure. Evidently, under the conditions described, all three substances acted as vasodilators. If, on the other hand, these substances are injected into the Locke's solution perfusing the vessels of the rabbit ear, the perfusion having continued for 24 hours, then each substance causes vasoconstriction. It is therefore clear that all three substances can act either as vasoconstrictors or as vasodilators, according to the conditions in which they are applied.

Some evidence suggests that the vasodilator effect of adrenaline, which has been observed only *in vivo*, may not be due to adrenaline itself but to the release of histamine by adrenaline. Thus Staub (1946) has shown that the injection of adrenaline causes a rise in the amount of histamine in the blood. It seems now reasonably certain that adrenaline has a direct vasodilator action of its own. The observation of Rothlin (1925), that in

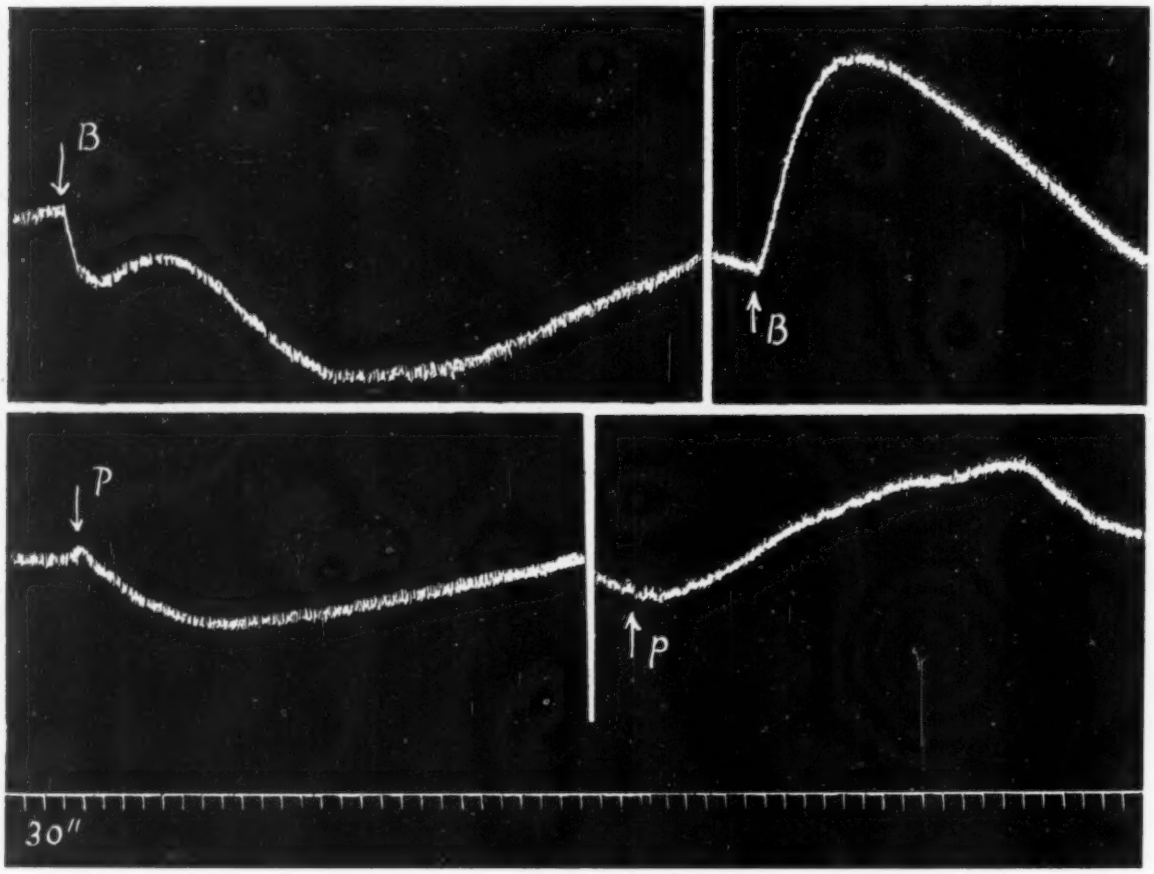

FIG. 6.—Upper tracings show action of benadryl (B): 100 μg. benadryl produced constriction; during perfusion with priscol (200 μg./ml.), benadryl (10 μg.) produced dilatation. Lower tracings show action of pethidine (P): 2 μg. pethidine caused vasoconstriction; during perfusion with priscol, pethidine (10 μg.) produced dilatation.

the presence of ergotamine adrenaline dilates the vessels of the perfused rabbit ear, has been confirmed by us using ergotoxine, and our colleague Gowdey (1948) has observed that in the presence of priscol adrenaline dilates the vessels of the rabbit ear. During priscol infusion the constrictor action of histamine itself was reduced but never reversed, so that the dilatation caused by adrenaline could not be due to histamine. More important, however, is our observation that in two fresh preparations adrenaline produced vasodilatation without the injection of any reversing agent. In one of these the vessels responded by dilatation during 8 hours' observation not only to adrenaline but to histamine and acetylcholine as well; no other substance than these three was injected at any time; the perfusion with Locke's solution was continued during the night, and on the following morning the response to the injection of adrenaline, histamine, and acetylcholine had become constrictor. In the second preparation the injection of adrenaline caused an initial constriction followed by dilatation; this was also observed several times. In the course of 20 preparations a vasodilator action of acetylcholine has almost always been observed during the early stages of perfusion; but repeated vasodilator responses to adrenaline and histamine have been seen in two preparations only.

There is, as Bülbring and Burn (1948) have found, a steady change in the response of the perfused vessels of the rabbit ear to the injection of acetylcholine; the response, which is at first dilator, becomes constrictor. There may be a similar change in the response to adrenaline, for although the initial response is almost always constrictor the vessels become much more sensitive with continued perfusion so that at a later stage a given dose produces more constriction than before. We have observed that this increased

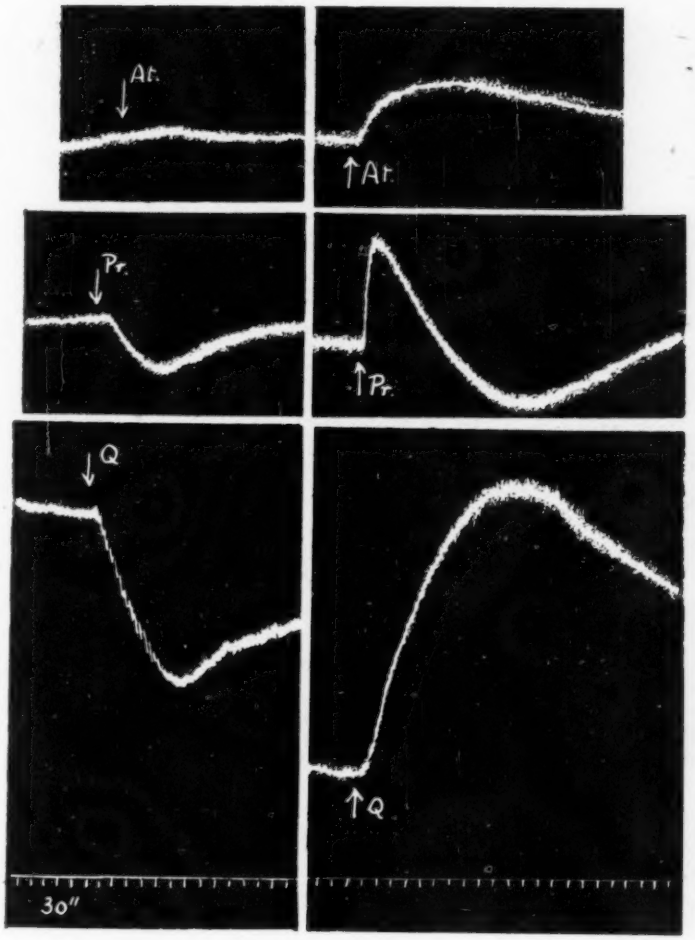

FIG. 7.—Similar to Fig. 6. Upper tracings 50 μg. atropine (At) before, and 100 μg. atropine during, priscol infusion. Middle tracings 500 μg. procaine (Pr) before and also during priscol infusion. Lower tracings 200 μg. quinidine (Q) before, and 100 μg. during, priscol infusion.

sensitiveness is reduced by perfusing a steady concentration of acetylcholine through the vessels, so that we do not think that the increased sensitiveness is due to oedema.

The view that the dilator effect of adrenaline or histamine is exerted on a different part of the vessels from that where these substances produce vasoconstriction has long been held ; such a view is less easy to hold when the system of vessels is as simple as that of the perfused rabbit ear. It seems at least equally probable that both effects are produced at the same site. Adrenaline stimulates the smooth muscle of the isolated rabbit uterus, but in the presence of ergotoxine adrenaline causes relaxation ; in this tissue it is very unlikely that there are two sites of action and much more likely that contraction and inhibition are produced at the same receptors. Both stimulation and inhibition by adrenaline were observed by McSwiney and Brown (1926) in several other tissues in which the site of action for both effects was probably the same. Recently Burn and Vane (1948) have demonstrated that acetylcholine causes both stimulation and inhibition in the isolated rabbit auricles after treatment with paludrine, and also in the isolated rabbit intestine and the isolated rat uterus. These opposed effects presumably occur at the same site in each organ, and we therefore favour the view that the two effects of adrenaline in blood vessels are also produced at the same site.

The evidence is stronger that the dilator and constrictor effects of histamine are produced at different sites, though Burn and Dale (1926) observed that histamine caused dilatation of the perfused mesenteric artery of the dog with its fine arterial branches, thus showing that dilatation is not produced by histamine in capillaries only.

Our observations also indicate the close relation which exists between the action of acetylcholine and that of adrenaline on the blood vessels. Substances such as atropine, pethidine, quinidine, benadryl, and procaine, which inhibit the constrictor action of acetylcholine, also inhibit the constrictor action of adrenaline in the rabbit ear. The close relation is also shown by the observation that the perfusion of priscol through the vessels reversed not only the constrictor action of adrenaline but that of acetylcholine as well. Vasodilatation thus produced by adrenaline was reduced or abolished by a preceding injection of

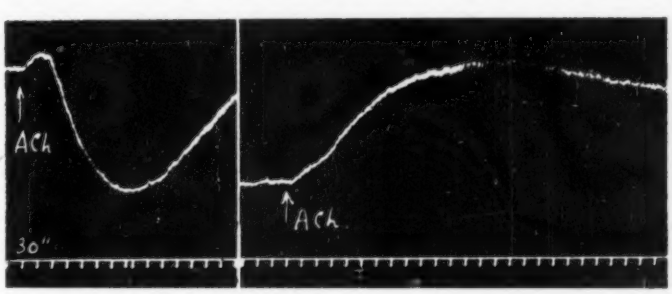

FIG. 8.—Reversal of constrictor action of acetylcholine by priscol. 10 μg. acetylcholine before, and 100 μg. acetylcholine during, priscol infusion.

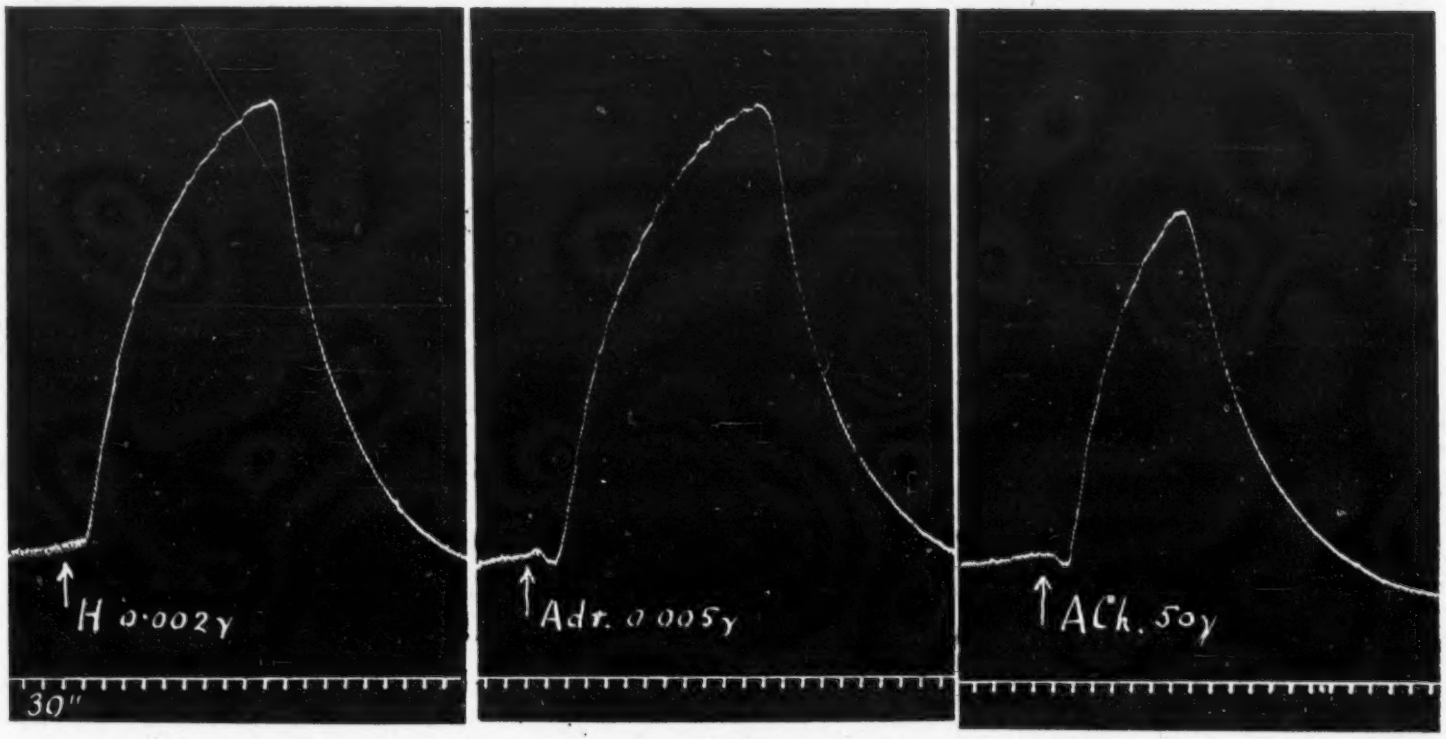

FIG. 9.—Exceptional dilator effects of histamine acid phosphate, 0.002 μg. (H), and of adrenaline hydrochloride, 0.005 μg. (Adr), in freshly prepared ear. The usual dilator effect of acetylcholine chloride, 50 μg. (Ach), is also shown. After 24 hours all three substances caused vasoconstriction in this preparation.

substances like benadryl, pethidine, and atropine.

Möller (1937) has observed that procaine constricts the vessels of the rabbit ear, and we found that large doses of benadryl, pethidine, and quinidine also cause vasoconstriction; these constrictor effects are reversed like that of adrenaline by perfusing the vessels with priscol. It was surprising to observe that quinidine reversed the vascular action of adrenaline like ergotoxine. We performed the experiment because Keogh and Shaw (1943) described an adrenaline reversal in the cat under nembutal anaesthesia following the injection of quinine. Having observed an adrenaline reversal by quinidine in the vessels of the rabbit ear, we proceeded to test the other substances in the same way, and found that when these are perfused in constant concentration through the vessels, the constrictor action of adrenaline is reversed by benadryl, procaine, and even pethidine, though the initial phase of the constrictor action is not completely abolished by pethidine. The substances which reverse the action of adrenaline have been hitherto regarded as a group apart, but our findings indicate that the properties first described for ergotoxine are properties shared by substances as dissimilar as quinidine and benadryl.

These additional observations that benadryl, procaine, pethidine, and quinidine are themselves constrictor substances which, like adrenaline or acetylcholine, are reversed in their vascular action by priscol suggests that when they inhibit the action of adrenaline or acetylcholine they do so because of their adrenaline-like or acetylcholine-like property; that is to say, the inhibition is due to competition for the same receptors.

If it is true that they compete for the same receptors, how is their ergotoxine-like property to be explained? The constrictor action of adrenaline is, of course, a motor effect, and the dilator action after ergotoxine is an inhibitor effect. Burn and Vane (1948) have suggested that with acetylcholine the transition from a motor to an inhibitor effect always occurs when excess of it is present. Such an explanation may apply to adrenaline. If the addition of benadryl or pethidine simulates the addition of a maximal amount of adrenaline, then the further addition of adrenaline would cause inhibition.

Summary

1. Earlier work has shown that a group of substances which includes atropine, benadryl, pethidine (demerol), procaine, and quinidine have the common property of antagonizing the action of acetylcholine. Experiments on the perfused vessels of the rabbit's ear have now shown that these substances will also inhibit the constrictor action of adrenaline and sympathetic stimulation.

2. When the constrictor action of adrenaline is reversed by priscol, these substances, which inhibit the action of acetylcholine, also inhibit the vasodilatation caused by adrenaline.

3. These substances, when perfused through the vessels, exert an action like that of ergotoxine or priscol; they reverse the constrictor action of adrenaline.

4. These substances can themselves exert a constrictor action on the vessels, and this constrictor action, like that of acetylcholine seen in the rabbit's ear vessels after 24 hours, is reversed under the influence of priscol.

5. In occasional fresh preparations, the injection of adrenaline regularly causes vasodilatation; though after several hours' perfusion with Locke's solution, the injection causes vasoconstriction.

6. The constrictor action of histamine in the rabbit ear vessels is abolished by the substances which abolish that of adrenaline. Priscol does not reverse the action of histamine. In occasional fresh preparations, histamine causes vasodilatation.

7. The view that adrenaline has a dilator action of its own is discussed.

8. The view that adrenaline dilatation and constriction are produced at the same site is discussed.

9. The similarity in the action of adrenaline and acetylcholine is emphasized.

10. The evidence is considered to support the theory of drug action by competition, and to suggest the mechanism of adrenaline reversal.

This work was carried out by one of us (N. K. Dutta) during the tenure of a grant from the Health Department, Government of India.

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